HORMONE

Einführung in ihre Chemie und Biologie

Von

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Den Abschnitt über Analyse der Steroidhormone in biologischem Material bearbeitete Ph. Mr. Oksana Sidlíková, Prag

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Vorwort

Die Entwicklung der Hormonforschung steht vor allem mit den Problemen der praktischen Medizin in engem Zusammenhang. Daher behandeln auch die meisten Werke die Hormone vom rein physiologischen und therapeutischen Standpunkt aus. In der Weltliteratur gibt es nur wenige Bücher, die sich vorwiegend mit der Chemie der Hormone beschäftigen. In dem vorliegenden Werk werden die einzelnen Hormone eben von diesem Gesichtspunkt aus betrachtet; damit jedoch der Stoff möglichst abgerundet dargestellt werden konnte, wurden auch Abschnitte über Biologie und Verwendung der Hormone einbezogen. In dem einführenden Kapitel werden die Grundbegriffe der inneren Sekretion besprochen, ohne die der weitere Text für Leser ohne medizinische Vorbildung nicht hinreichend verständlich wäre.

In diesem Buche sind vor allem die Erkenntnisse über die *Hormone der höheren Wirbeltiere* zusammengetragen, und die Einteilung erfolgte nach chemischen Gesichtspunkten in drei Hauptkapitel, d. h. in die von der Aminosäure Tyrosin abgeleiteten Hormone, die Steroidhormone und die Hormone mit Peptidstruktur. In dem einführenden Kapitel findet sich ein kürzerer Abschnitt über die *Hormone der Wirbellosen.* Die sogenannten Pflanzenhormone wurden in dem vorliegenden Buche nicht mit aufgenommen, da diese Stoffe nicht einmal in physiologischer Hinsicht mit den wirklichen Hormonen in näherem Zusammenhang stehen. Ihre Bezeichnung war ziemlich willkürlich nach der oberflächlichen Analogie gewählt worden, ohne daß eine Parallelität zwischen dem Steuerungsmechanismus bei Tieren und Pflanzen besteht; noch weniger kann allerdings von einer inneren Sekretion bei den Pflanzen gesprochen werden.

Diese Einführung in die Chemie und Biologie der Hormone soll grundlegende Aufschlüsse namentlich über die Problematik der *Chemie* und des *Stoffwechsels* dieser Substanzen geben, ohne auf die komplizierten und bisher oft noch nicht zufriedenstellend gelösten Probleme der Wechselbeziehungen der endokrinen Organe, der Einreihung des endokrinen Systems in das Gesamtsteuerungssystem des Organismus und seines Zusammenhanges mit dem Nervenapparat näher einzugehen. Diese Probleme stehen vor allem den Endokrinologen zu.

Die Literatur über die Hormone ist heute bereits so umfangreich, daß ein Band allein nicht einmal ihre bloße Aufzählung umfassen würde. Bei den einzelnen *Abschnitten dieses Buches* sind die Literaturangaben vor allem auf die wichtigsten Arbeiten ausgerichtet; dennoch mußten einige tausend Veröffentlichungen an-

geführt werden. Diese Literatur ermöglicht dem Leser das Auffinden von Einzelheiten, insbesondere in methodischer Hinsicht, auf die hier nicht eingegangen werden konnte. Die Literatur wurde bis Ende des Jahres 1957 bearbeitet, von den späteren Arbeiten wurden nur die wesentlichsten herausgegriffen, soweit sie zur Zeit der Bearbeitung des Manuskriptes zur Verfügung standen.

Der Text der ersten Ausgabe dieses Buches aus dem Jahre 1953 wurde vollständig umgearbeitet; denn während der letzten 5 Jahre konnten viele Erkenntnisse auf dem Gebiete der Hormone gesammelt werden, die oft die frühere Auffassung völlig veränderten. Der Abschnitt über Kallikrein entfiel, da sich das Präparat als Enzymgemisch erwiesen hat. Das Buch enthält einen neuen Abschnitt über Relaxin. Die Abschnitte über den Stoffwechsel der Steroidhormone sowie der Abschnitt über die Nebennierenrindenhormone wurden wesentlich erweitert. Um den Umfang des Buches nicht zu sehr zu vergrößern, mußten manchmal beinahe schlagwortartige Formulierungen gewählt werden. Eine kurzgefaßte historische Entwicklung der Erkenntnisse wurde bei den einzelnen Hormonen belassen, wogegen alle überholten Anschauungen wegfielen. Bei der Struktur der Hormone sind die endgültigen Befunde ohne ihre Ableitung angeführt, da dies über den Umfang des vorliegenden Buches hinausginge. Ähnlich verhält es sich bei den synthetischen Herstellungsverfahren, wo nur das Prinzip des Verfahrens angedeutet ist. Die Literaturangaben ermöglichen es jedoch dem Leser, alle notwendigen Einzelheiten nachzuschlagen.

Mein besonderer Dank gebührt dem VEB Gustav Fischer Verlag in Jena, der die Herausgabe dieses Buches in deutscher Sprache nach der 2. umgearbeiteten tschechischen Auflage ermöglichte, ferner Frau Dr. Riedl-Tůmová für die sorgfältige Übersetzung und meinen Mitarbeitern aus dem Forschungsinstitut für Pharmazie und Biochemie in Prag für wertvolle Anregungen und Mithilfe bei der Abfassung des neuen Manuskriptes. Den Abschnitt über die Analyse der Steroidhormone im biologischen Material hat Frau Ph. Mr. Oksana Siblíková sehr sorgfältig separat bearbeitet.

Prag, im Juni 1959

Dr. Ing. Oldřich Hanč

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I. Einleitung

1. Innere Sekretion und Endokrinologie

Das zweckmäßige Zusammenwirken der Gewebe und Organe der höheren Organismen kommt auf zwei Wegen zustande, und zwar — schematisch ausgedrückt — *nervös* und *humoral*. Die einzelnen Zellen in ihrer Gesamtheit, das sind Gewebe und Organe, beeinflussen durch ihren Stoffwechsel auch die übrigen Teile des höheren Organismus, welcher mit einer Reihe regulierender Einrichtungen ausgerüstet ist (42), deren Aufgabe es ist, ein Entgleisen des Stoffwechselgleichgewichtes zu verhindern. Die für den Fortbestand des Organismus unerläßliche Aufrechterhaltung dieses Gleichgewichtes erfolgt durch viele gleichzeitig verlaufende Reaktionen, deren Ablauf durch thermodynamische Gesetzmäßigkeiten von allgemeiner Gültigkeit für das Naturgeschehen bedingt ist. Es ist besonders auf das LE CHATELIERsche Prinzip von der Erhaltung des beweglichen Gleichgewichtes aufmerksam zu machen, nach welchem ein System unter dem Einfluß der äußeren geänderten Bedingungen derart reagiert, daß es unter den gegebenen Umständen den ursprünglichen Zustand der optimalen Bedingungen beibehält. Diese „Autoregulation" gilt sowohl für „nicht organisierte" Systeme, z. B. die chemische homogene und heterogene Phase, wo sie auf gröbere Art zur Geltung kommt, als auch für „organisierte" Systeme, wie z. B. Zellen, Gewebe, Organe und schließlich Gesamtorganismen. In der angedeuteten Reihe der organischen Einheiten ist die zunehmende Empfindlichkeit in der Autoregulation gegenüber Einflüssen der Umgebung charakteristisch; diese wird eben durch die organische Spezialisierung der einzelnen Funktionen bedingt, welche allerdings eine Verengung des Existenzbereiches einer derartig spezialisierten organischen Einheit mit sich bringt.

Die Einteilung in nervöse und humorale Regulation des Organismus ist natürlich sehr grob und willkürlich; sie ist zwar in morphologischer Hinsicht anschaulich, stellt jedoch nicht den grundsätzlichen Unterschied im biologischen Mechanismus des Reizes und seiner Übertragung dar (45, 56, 59, 68, 111, 122). Die Bedeutung des Acetylcholins, Adrenalins usw. für die Reizübertragung im Nervensystem und den entsprechenden Körperorganen wird heute allgemein anerkannt (107). Die Nervenreflexe werden nämlich auch durch spezifisch wirkende Substanzen hervorgerufen und übertragen, die an der zugehörigen Stelle mit einem bestimmten Akzeptor in Reaktion treten und so eine Reihe von Reaktionen hervorrufen, deren Er-

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CHAPTER 1

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HERMANN PINKUS

Wayne State University, School of Medicine, Detroit, Michigan

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I INTRODUCTION

Data on the embryologic development of hair have been contributed by many authors for over 100 years Milestones in our knowledge of the ontogenesis of human hair are the work of Koelliker (1850), Unna (1876), and Stohr (1903-1904) to name just a few F. Pinkus (1910, 1927) described the development of the human pilary system in detail in Keibel and Mall's classical text and again in Jadassohn's Handbuch English language descriptions are available in the writings of Danforth (1925) and Trotter (1932). Recent texts on human embryology have tended to treat the subject rather cursorily.

Development of hair in mammals has been investigated in a considerable number of families and species from the marsupials to the primates (Stohr, 1903-1904, Segall, 1918, Chase, 1954). Most recently, because of their practical use in the laboratory or in husbandry, rodents and sheep have been in the foreground of interest. The great lines of

development and even many details are similar in all mammals, but minor variations exist.

Little work has been done on the embryology of human hair with modern staining methods and under modern points of view. The observations which follow were made on well-preserved human fetuses ranging from 100–200 mm crown rump (CR) length, and fixed immediately in formol-alcohol and in formalin (Table I). Skin from the anterior portion of the scalp, the chest and the back of the trunk was stained with a variety of methods. The actual age of the fetus was of little concern since some smaller specimens showed more advanced development than larger ones. The pieces of skin were embedded in paraffin and serial sections of 10 μ thickness were stained with hematoxylin and eosin, hematoxylin and Van Gieson's acid fuchsin-picric acid mixture, acid orcein and Giemsa solution (Pinkus, 1944), toluidine blue at pH 6, thionine, and with the periodic acid-Schiff procedure (PAS) counterstained with light green In addition to morphological detail, attention was paid particularly to the process of keratinization at the various levels of the growing follicle, to the development of the mesodermal portions of the pilary complex, and to the quantities of glycogen present at different stages in the various epithelial components of the skin.

The following description, therefore, is based on personal observations carefully compared with data found in the literature. Only minor new details were observed, some of which are very important. New photomicrographs are used in the main for illustration, but since it is difficult to find ideal fields for photography of the major developmental phases, it was decided to reproduce the beautiful lithographic plates of Stohr's drawings (1903) for general reference purposes (Plates I–III).

II. Chronology

The first hair anlagen appear toward the end of the second or early in the third month in the regions of the eyebrows, upper lip, and chin. Keibel and Elze (1908) found pilary germs in the supraorbital region of an embryo of 27 mm total length. F Pinkus (1910) counted 170 anlagen in the eyebrow region, 130 on the upper lip, and a few on the chin of a 32-mm embryo, even more and somewhat further advanced stages in a 30-mm specimen In all other mammals, vibrissae are found in these sites and develop much earlier than hairs of the general pelage. While man has no hairs of the specific structure of animal vibrissae (sinus hairs) the sites of the first hairs are similar General development of hair does not begin until the fourth fetal month In any individual fetus, hairs are farthest advanced cephalad and the development spreads caudad. As will be described in Section IV, primary hair

TABLE I

HUMAN FETUSES ARRANGED ACCORDING TO DEVELOPMENT

No	Acc No	Crown rump length (mm)	Race	Fixative	Stage of hair development	Remarks
1	P-884	110	Negro	Formol-alcohol	Early	Numerous mitoses
2	P-880	110	Negro	Formol-alcohol	Early	Mitoses?
3	P-888	115	White?	Formol-alcohol	Rather early	Occasional mitoses, apocrine glands
4	P-887	135	Negro	Formol-alcohol	Intermediate	Apocrine glands
5	P-883	190	Negro	Formol-alcohol	Intermediate	Apocrine glands, some glycolysis
6	P-874	150	White	Formalin	Intermediate	Slightly macerated
7	P-878	180	Negro	Formol-alcohol	Advanced	
8	P-885	191	Negro	Formol-alcohol	Advanced	Somewhat hemorrhagic
9	P-882	150	Negro	Formol-alcohol	Far advanced	Occasional mitoses
10	P-875	200	White	Formalin	Farthest advanced	Elastic fibers

germs form more or less simultaneously at fairly even distances (Figs. 1, 2). As the skin grows, new primary germs develop between the earlier ones (Figs. 3, 14). Later secondary germs develop close to the primary ones and form hair groups (Figs 6, 9, 22, 24). Through these two mechanisms hair follicles in various stages of development may be found in the same region.

III. Morphology

A. Earliest Stages

1. *Pre-germ Stage*

The first sign of a hair follicle in the human skin is a crowding of nuclei in the basal layer of the epidermis (Figs. 9, 15). This is called

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Key to Lettering on Figs 1–46

A	arrector muscle	I	inner root sheath
A G	apocrine land	In	infundibulum
Bb	bulbus	Is	isthmus
Bg	bulge	L	lower follicle
B M	basal membrane	M	mitosis
C	hair canal	Ma	matrix
C L.	critical level of matrix	Mc	melanocytes
Cu	cuticle of inner sheath	M G	melanin granules
D	division between epidermal and follicular basal cells	O	outer root sheath
Du	duct of sebaceous gland	P	papilla
El	elastic fibers	P G	primary hair germ
E G.	eccrine gland	P P	papillary pad
F	fibrous root sheath	Seb	sebaceous gland
F T	fat tissue	S G	secondary hair germ
H	hair	V	vitreous membrane

Fig 1 Fetus 1, skin of chest Early hair germs Hematoxylin and eosin Magnification × 28

Fig 2 Fetus 1, scalp Hair peg stage. Hematoxylin and eosin Magnification × 28

Fig 3 Fetus 3, scalp Bulbous peg stage, young primary germs between the older ones Hematoxylin and eosin Magnification × 28

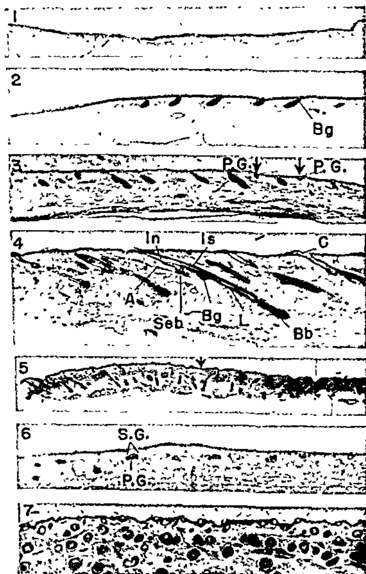
Fig 4 Fetus 9, scalp Fully developed primary follicles and shorter secondary follicles Hematoxylin and eosin Magnification × 28

Fig 5 Fetus 7, dorsal skin Reversal of hair stream, note position of hair bulbs in the fat tissue Hematoxylin and eosin Magnification × 11 2

Fig 6 Fetus 5, dorsal skin Cross sections of three-hair groups at various levels Magnification × 28

Fig 7 Fetus 10, scalp Cross sections of hair follicles and hair canals, some eccrine glands are present Three-hair groups become obliterated early on the scalp. Hematoxylin and eosin Magnification × 28

primitive hair germ or "pre-germ" (Haarvorkeim, F. Pinkus (1910)). At this stage, the epidermis often consists of only two layers, germinal cells and periderm, but it may show development of the third (intermediate) layer, and in the case of secondary follicles the epidermis usually is multilayered (Fig 9) and may show true keratinization. The events, however, are the same in all circumstances. The very early stage is



difficult to identify in sections. It is more easily seen in spreads of epidermis as shown by Fleischhauer (1953b). The nuclei of the stratum germinativum, and to a lesser degree those of the stratum intermedium become more numerous, are smaller, and stain darker in a round area about 8 to 12 cells wide (Figs. 14, 47). There has been considerable difference of opinion whether an accumulation of mesodermal nuclei precedes, accompanies, or follows the first visible changes in the epidermis The general opinion of authors seems to be that with human hair and the pelage of animals, epidermal alterations are first, while vibrissae and specialized structures such as the quills of the hedgehog may show a mesodermal precursor stage. Such anlagen usually can be recognized as slight elevations on the outer surface of the skin, while there is no such change with ordinary hairs.

In epidermal spreads (Fleischhauer, 1953b) there is seen around each young hair germ a first denser, then a lighter areola that soon disappears. Fleischhauer (1953b) assumes that at first neighboring cells are drawn into the hair germ and contribute to its substance (in-

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Fig 8 Fetus 1, skin of chest Early hair germ with beginning accumulation of mesodermal nuclei, three mitoses are indicated, the one farthest left is in anaphase, the other two are telophases, only one-half of each diaster is in focus Toluidine blue Magnification × 450.

Fig 9 Fetus 6, dorsal skin Pre-germ of secondary follicle develops close to base of hair canal of primary follicle Hematoxylin and eosin Magnification. × 348 75

Fig 10 Fetus 8, dorsal skin Young primary germ developing close to tip of hair canal of an older follicle, note sharp anterior border to right, extension of crowded nuclei at posterior border to left This is the earliest sign of the hair canal Hematoxylin and eosin Magnification × 450

Fig 11 Fetus 1, scalp Sagittal section of hair germ exhibiting characteristic asymmetry and anlage of dermal papilla Hematoxylin and eosin. Magnification × 277 5.

Fig 12 Fetus 6, dorsal skin Oblique section of hair germ with extension of crowded nuclei at posterior left margin Hematoxylin and eosin Magnification × 348 75

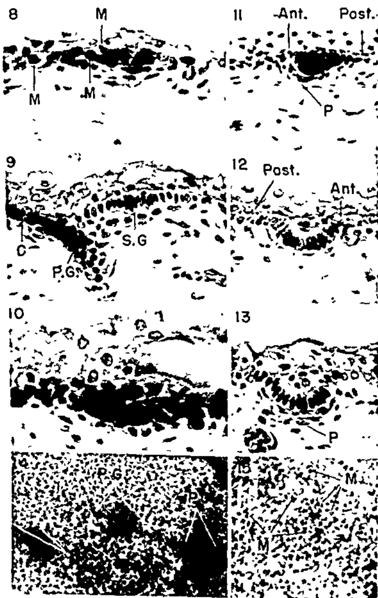
Fig 13 Fetus 5, dorsal skin Almost frontal section of hair germ Hematoxylin and eosin Magnification × 348 75

Fig 14 Fetus 2, skin of thigh, stained *in toto* with hematoxylin Young hair germ developing between two older ones, close to the tip of one hair canal Magnification × 135

Fig 15 Fetus 1, skin of arm, stained in toto with hematoxylin Pre-germ with one mitosis in center and several others at periphery. Magnification × 138 75

For key to lettering see legends to Figs 1–7

vagination), while the follicle later grows by mitotic division of its own cells. The presence of more numerous mitoses in the immediate surroundings of primitive hair germs supports this thesis (Fig 15). A slightly older germ exhibiting three mitotic figures is illustrated in Fig 8.



2. *Hair Germ Stage*

The pre-germ passes rapidly into the *hair germ* stage (Haarkeim, Stohr, 1903). The basal cells now become very high, the nuclei elongated, and the entire structure protrudes downward into the corium (Figs. 10–13) Additional cells accumulate above the basal layer, beneath the periderm. The peculiar arrangement of the epithelial nuclei in this stage (Figs. 10, 12) was compared to the arrangement of logs in a charcoal kiln (Meiler) by Maurer (1892). This comparison has become meaningless in modern times. While in young embryos the entire epidermis including the basal layer is laden with glycogen, the hair germ cells rapidly lose this substance and therefore stand out clearly in sections treated with PAS. As glycogen disappears, a basement membrane becomes visible as a sharply defined red line (Figs. 16, 17). At this stage, accumulation of relatively small and dark staining mesodermal nuclei is obvious around the bulge of the basal layer (Figs. 10–13, 16).

The hair germ exhibits *bilateral* symmetry at an early stage. Hair germs, therefore, will present different aspects depending on whether they are sectioned in a transverse or sagittal direction (Figs. 11-13). In a given section, practically all germs will offer similar pictures (Figs. 2-4, 6, 7). It seems most advantageous to cut sections so that they show the whole length of hair follicles. Such sections show a characteristic asymmetry of early germs (Fig. 11) One side is steep and forms a right angle with the epidermis, the other side slants and merges with basal layer gradually. As the germ develops it grows

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Fig 16 Fetus 1, dorsal skin Epidermal details obscured by glycogen Hair germ has lost most glycogen, exhibits basal membrane at lower pole and dense accumulation of mesodermal nuclei PAS Magnification × 480

Fig 17 Fetus 5, pectoral skin This specimen exhibited signs of glycolysis. Cross section of primary follicle and two secondary germs showing basal membrane Magnification × 320

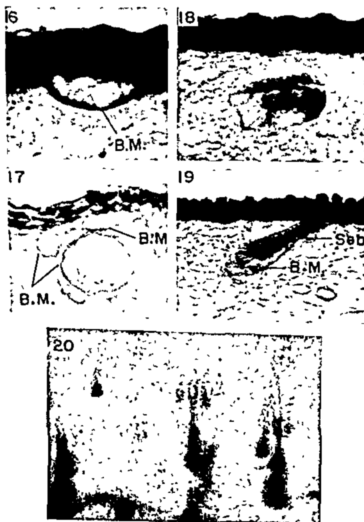
Fig 18 Fetus 1, scalp. Hair peg showing slightly concave contour of advancing border and definite basal membrane Fanlike matrix free of glycogen Cells of hair peg contain some glycogen Epidermal basal layer begins to loose glycogen PAS Magnification × 320

Fig 19 Fetus 1, scalp Bulbous peg with early differentiation of sebaceous gland and bulge Sebaceous gland forms an almost glycogen-free knob at posterior side of follicle Bulge, matrix, and papilla are cut somewhat tangentially. PAS Magnification × 180

Fig 20. Specimen obtained from Dr A A Zimmermann Fetus 99, skin of thigh, 4 8 months, stained *in toto* with silver Three-hair groups show distribution of melanoblasts at basal layer of external root sheath Magnification × 72

For key to lettering see legends to Figs 1–7

obliquely downward, converting the right angle into an acute one and foreshadowing the slant of the fully formed hair (Figs. 2, 18). F. Pinkus introduced the terms "anterior" and "posterior" into the description of the pilary system. These terms are based on the hypothesis that the hair is formed and emerges "behind" a scale, a condition that actually prevails on the tail of mammals. More will be said about this in Section IV. F. Pinkus' terminology offers a number of advantages in describing the development of the pilary complex. It is easy to remember



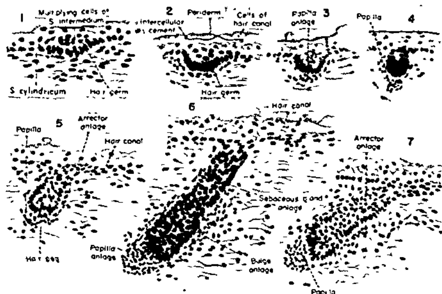


Plate I. Photographic reproduction of lithographic Plates III/IV from Stohr (1903) English legends substituted Reduced from original 11½ × 7¾ inch size.

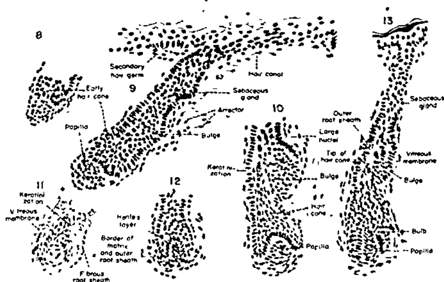


Plate II Reproduction of Plates V/VI from Stohr (1903), reduced

that the part of the skin that is shaded by the slanting hair shaft is behind (posterior to) the follicle.

3. Hair Peg Stage

From its point of origin the hair germ grows obliquely down and forward into the mesenchyme in the shape of a solid column of epithelial cells that seems to push a cluster of mesodermal nuclei before it (Plates I, 6-7, II, 8-9). The outer cells of the *hair peg* (Haarzapfen, Stohr (1903)) are columnar and arranged radially to the long axis (Figs 19, 24). Cells in the center first have no definite polarity, but soon become arranged longitudinally. The highest cells always are those of the advancing end, destined to become the matrix The advancing end is the broadest part of the peg and forms either a straight transverse plate or is slightly concave because of pressure against the compact ball of mesodermal cells, the future dermal papilla (Fig. 18). The entire column is enveloped in a sheath of mesodermal cells contiguous with those of the papilla. Zimmermann (1953) has shown that melanoblasts are present on the outer surface of the epithelial peg (Fig 20). As the young follicle becomes elongated, first its central cells and later the peripheral basal cells begin to acquire glycogen again, with the exception of the tall matrix cells which always remain free of it (Fig 24)

B Beginning of Differentiation

1 Bulbous Peg Stage

As the follicle continues to grow longer, differentiation sets in. The advancing border enlarges, becomes bulbous and envelops part of the mesodermal material which now is divided into the egg-shaped papilla inside the hollowed-out bulb of the matrix and the papillary pad below the bulb Papilla and pad are connected by a gradually narrowing neck (*bulbous peg*, Bulbuszapfen, Stohr, 1903). Simultaneously, two solid epithelial swellings develop at the posterior side of the follicular column. The lower one always remains solid and its cells grow rich in glycogen together with the rest of the follicle. This structure was named "Wulst" (bulge) by Stohr (Plate II). It may be so inconspicuous in adult skin that many textbooks of histology do not mention it. It is very conspicuous in embryonic life, and often develops earlier and is larger than the more superficial swelling that is the anlage of the sebaceous gland In contrast with the bulge, the sebaceous gland anlage is free of glycogen at this stage (Fig 25) It is a rounded knob (Fig 22) the central cells of which soon accumulate lipid and appear foamy in paraffin sections (Fig 23) A solid cord of elongated cells extends backward within the epidermis (Figs 21-22), and represents the anlage of the *hair canal*

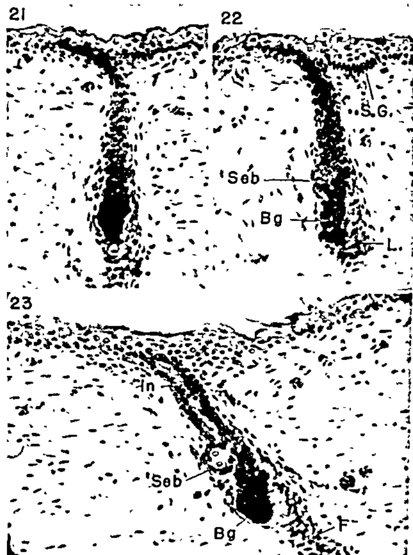


FIG 21 Fetus 6, dorsal skin Bulbous peg with early hair canal, bulge, and secondary hair germ Of the lower follicle only the fibrous root sheath is cut Hematoxylin and eosin Magnification × 224

FIG 22 Fetus 6, dorsal skin Similar follicle showing early sebaceous gland anlage above the bulge Hematoxylin and eosin Magnification × 224

FIG 23 Fetus 6, dorsal skin Further advanced follicle with a few lipidized cells in the sebaceous gland and keratohyalin granules and early keratinization in the infundibulum The fibrous root sheath of the lower follicle is seen extending downward beyond the bulge Hematoxylin and eosin Magnification × 224

For key to lettering see legends to Figs 1–7

(Haarkanal); this is a transient but regularly present fetal differentiation The last constant part of the pilary complex becomes visible at a slightly later stage. At a little distance from the sebaceous gland, free in the mesenchyme, mesodermal cells arrange themselves in a slender row, parallel to the posterior border of the follicle This region is the first and most conspicuously metachromatic area of the corium in sections stained with basic dyes Thus the musculus arrector pili is formed. It gradually extends downward toward the bulge. Up to this point, the follicle is a solid epithelial structure surrounded by a mesodermal sheath.

C. *Differentiation of Various Parts*

With all the components of the pilosebaceous follicle now apparent, growth and differentiation continues and seems to be peculiarly self-regulated though coordinated in the various portions. Seven segments can be recognized.

There is (1) the *bulb* comprising the matrix of the hair and its sheaths and the mesodermal papilla. (2) The *lower follicle* from the upper end of the bulb to (3) the *area of the bulge.* Between the bulge and the sebaceous gland there is a shorter or longer stretch called (4) the *isthmus.* This is followed by (5) the *area of the sebaceous* gland. From the point of its opening into the follicular lumen to the base of the epidermis we have (6) the *infundibulum,* which continues within the epidermis as (7) the *hair canal.* All of these segments eventually are traversed by (8) the *hair and inner root sheath.* In addition (9) the *arrector muscle* and (10) the *apocrine gland* need consideration.

1 *The Bulb*

The lower bulbous end of the follicle harbors the mesodermal papilla which consists of closely packed cells (Fig 26 and Plate III) No metachromatic intercellular substance is present in the papilla up to the latest stages examined in my series even though hairs are fully developed Only in the second oldest fetus some metachromasia was seen in the papillae of scalp hairs The oldest fetus had been preserved in formalin and metachromatic staining could not be demonstrated in any part of its skin The PAS procedure reveals some pink staining fibrillar material between the papillary cells in moderately advanced stages and always shows a definite fine red line between matrix and papilla from the earliest stage on (Figs. 18, 19). This line is obscured only when melanocytes are present in the matrix. Scattered granules of glycogen often are present in the cells of the papilla, with even more in the subpapillary pad.

The cells of the matrix are always free of glycogen. They are tall

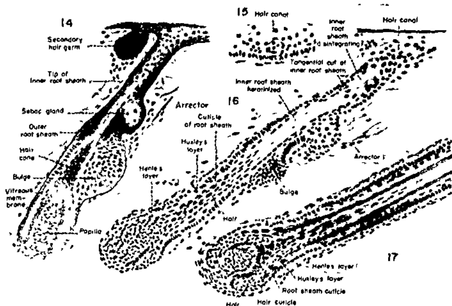


PLATE III Reproduction with substituted English legends of colored lithographic Plates VII/VIII from Stöhr (1903). Original size 11½ × 7¼ inches

columnar in young stages and arranged somewhat fan-shaped (Figs. 18, 24) Later, densely packed smaller cells surround the papilla. While these cells seem to form an undifferentiated mass in most staining procedures, staining with PAS shows that glycogen-containing cells of the outer root sheath extend down to the lowest extremity of the follicle as a

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FIG 24 Fetus 1, scalp Frontal section of the furthest advanced follicle seen in this specimen with one early secondary germ on either side. Large amount of glycogen present in root sheath, none in matrix PAS Magnification. × 180

FIG 25 Fetus 7, dorsal skin Curved hair follicle extends into the subcutaneous fat and exhibits sharp difference between the glycogen-filled lower follicle and bulge and the glycogen-free matrix, hair, and upper segments Loss of glycogen in the epidermal basal layer PAS Magnification × 72

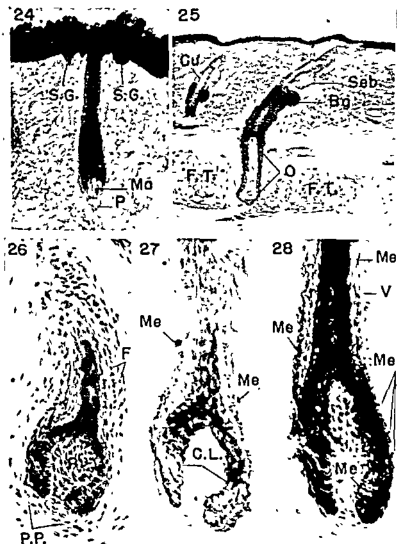
FIG 26 Fetus 7, scalp Hair bulb with papilla and matrix and deeply pigmented hair Some melanocytes are visible along the basal layer of the outer root sheath Hematoxylin and eosin Magnification × 224

FIG 27 Fetus 9, scalp Hair bulb contains numerous melanocytes above the critical level of the matrix Scattered melanocytes in lower part of matrix and outer root sheath Toluidine blue Magnification × 295

FIG 28 Fetus 9, scalp Similar follicle with melanocytes Vitreous membrane appears as a clear space between the outer root sheath and the fibrous sheath Toluidine blue Magnification × 295

For key to lettering see legends to Figs 1–7

thin outer coat (Fig 25). Round melanocytes are found between the epithelial cells of the Negro fetus even before the bulb is fully formed. Later, numerous large dendritic cells laden with pigment granules are present in the upper half of the bulb (Fig. 27), above the "critical level", described by Montagna (1956) as the border between indifferent matrix and differentiating cells of hair and root sheath. In contrast to adult conditions, there are also scattered melanocytes in the lower half of the bulb and in the outer root sheath for some distance above



the bulb (Figs. 27, 28). The bulb is surrounded by numerous mesodermal cells, the beginning of the connective tissue sheath. They become particularly prominent around the lower end where they form a thick pad (Fig. 26). Capillary blood vessels approach the follicle near its lower end and lie between the fibroblasts of the pad and around the bulb, but in spite of close search, none was seen to be present within the papilla. The border between mesodermal and ectodermal cells, always outlined by a thin PAS-positive membrane, soon becomes accentuated by the beginnings of the vitreous membrane which will be described in more detail in Section III, C, 2 It usually can be followed to the lowest point of the external root sheath (Plate III, 14). In older specimens, it seems to be continuous with PAS-positive fibrillae entering the papilla The shape of the bulb usually is asymmetrical in sagittal sections of the follicle The neck of the papilla points toward the subcutis and the anterior upper rim of the matrix is longer than the posterior lower one

2 *The Lower Follicle*

Above its bulbous end the follicle is a cylindrical structure consisting of stratified epithelium. Solid at first, it later has a central lumen containing the hair and its sheaths It then has become the outer root sheath. In young stages, radially arranged columnar cells form a basal layer (Plate II, 9) around poorly oriented central cells, which soon become elongated and arranged longitudinally, even before the internal root sheath pushes up from the matrix These central cells practically always contain some glycogen The basal cells soon acquire more and more and eventually are so full of the substance (Figs. 25, 29, 30) that only a framework of cytoplasm remains Thus, they appear light and

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FIGS 29, 30 Fetus 9, scalp Details of glycogen distribution in bulge, isthmus, sebaceous gland, and infundibulum of fully developed follicles. Note cell-sharp boundary between glycogen-free epidermal basal cells and cells of infundibulum in Fig 29 Dark granules in Huxley's layer are melanin PAS Magnifications × 144

FIG 31 Fetus 7, dorsal skin Strongly curved hair follicle with early kink between bulb and lower follicle Carina separating follicular tube from sebaceous duct Arrector muscle inserts at shelflike upper border of bulge Toluidine blue Magnification × 72

FIG 32 Fetus 7, scalp Duct of sebaceous gland Melanin sludge in infundibulum and remnant of hair canal the roof of which has largely disappeared Part of arrector muscle extends beyond bulge Hematoxylin and eosin Magnification × 108

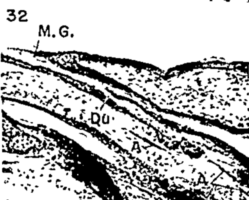
For key to lettering see legends to Figs 1–7

empty with all other stains (Figs. 4, 31, 40). By this time several layers of prickle cells have developed within the tube of basal cells and form a solid covering for the hair. No keratohyalin or any evidence of keratinization is observed in the lower follicle.

The mesodermal sheath soon shows arrangement of nuclei in two directions and fine fibrils form the circular and longitudinal layers of the fibrous root sheath (Fig. 26, Plate III, 17). These fibrils stain red with PAS and with acid fuchsin. They are young collagen fibrils Elastic fibers appear much later and were seen only in the furthest advanced specimen. At the junction of ectoderm and mesoderm a hyaline mem-







brane soon appears and may become 2–3 μ thick. This glassy or vitreous membrane stains pink with eosin in hematoxylin-eosin and orcein-Giemsa preparations (Figs. 28, 40). It is pale blue to purplish green in PAS slides counterstained with light green and seems to possess very thin outer and inner red limits in some specimens It is pale blue and not metachromatic with toluidine blue and thionine. The acid fuchsin of Van Gieson's mixture shows instead skeins of thin and sharply stained fibrils which seem to form a fenestrated membrane as illustrated by Stohr (1903-1904) in similar preparations.

3. *Area of the Bulge*

Above the cylindrical tube of the lower follicle the pilary complex again exhibits its bilateral symmetry. There is a projecting mass of epithelial cells at its posterior circumference. This *bulge*, often the most conspicuous detail of the young germ, is as large as the bulb and is often larger than the sebaceous gland (Figs 3, 23, 25). It is hemispherical, or it may have a slanting lower end and a shelf-like upper contour (Figs. 32, 41). It often reaches somewhat around the follicle and causes the anterior wall also to be slightly thickened (Fig 30). It consists of more or less radially arranged large epithelial cells containing almost as much glycogen as the outer root sheath, but never exhibiting rarefaction of the cytoplasm. The vitreous membrane extends around the bulge and ends abruptly at its upper limit. The function of the bulge is obscure. While it serves as the point of insertion of the arrector muscle later in life, it develops much earlier than the muscle and the latter seems to originate quite independently in the skin near the sebaceous gland, and in many instances streaks by the bulge before approaching the lower follicle below it. Unna (1876) named the bulge area of the adult follicle the *hair bed* (Haarbeet) believing that the club hair became implanted there and derived additional growth from it Stohr gave it the neutral name "Wulst" (bulge or swelling) Some texts state that this is an area of marked proliferative activity, but no mitotic figures were observed in the bulge even if other parts of the follicle contained them Whatever its function, the bulge marks the lower end of the "permanent follicle" later in life Everything below it is expendable during hair change (Montagna, 1956)

4 *The Isthmus*

The part between the bulge and the sebaceous gland has been called the *isthmus*. It is a border zone peculiarly devoid of specific features The vitreous membrane does not cover it After this segment has been hollowed out by the advancing hair, the posterior wall consists of two

or three rows of flattened cells (Fig 41) the nuclei of which exhibit a characteristic change of direction (Figs. 29, 30). Those of the lower part slant from the inside up and out and the cells resemble those of the bulge. The nuclei of the upper part of the isthmus slant downward and those in between are quite flat and parallel to the axis of the follicle The cells are more or less devoid of glycogen and contrast with those of the bulge and lower follicle (Fig 25). The transition is not quite so abrupt along the anterior wall, but the cells also are less high and contain much less glycogen The nuclei of the central cells of the isthmus *become pyknotic before the tip of the root sheath reaches this* area, but true keratinization is absent.

5 *The Sebaceous Gland*

The *sebaceous gland* at first is a solid hemispherical knob at the posterior wall of the follicle (Figs 22, 43). Often it is somewhat smaller than the bulge and practically always devoid of glycogen. Soon the central cells, often just one or two, begin to show the typical foamy structure of the cytoplasm that indicates the accumulation of lipid (Fig 23) As the gland gets larger its basal cells begin to contain some glycogen (Figs. 29, 30). The number of lipidized cells increases and *cells of this type now form the inner lining of the posterior circumfer*ence of the hollowed-out follicle (Figs 30, 44) The anterior wall is lined by keratinizing cells of infundibular character Thus at first, the sebaceous gland has no duct. Only at a later stage, when the gland already begins to show lobulation, do we see a carina-like septum dividing the follicle into the tube for the hair and into a short keratinizing tube leading to the sebaceous gland (Figs 31, 32)

6 *The Infundibulum and the Hair Canal*

The uppermost portion of the follicle is the first structure in the developing skin to contain keratohyalin granules and keratin (Figs 23, 43). This happens before the hair is formed and before the sebaceous gland has matured enough to require an outlet This portion may be subdivided into a subepidermal part, the infundibulum, and an intraepidermal part, the hair canal. The division is didactic rather than real as both parts have a similar structure and develop simultaneously

It is important to keep in mind that the point at which the epidermis *and the follicle form an acute angle, that is the junction of anterior* follicular wall and epidermal stratum basale, is the sole fixed point of reference in the topographical development of the pilary complex. This point marks the sharp anterior boundary of the early hair germ The posterior boundary usually is much less distinct (Figs 11, 12), and as

the hair bud grows slanting forward in the corium, a solid strand of cells grows backward in the epidermis (Figs. 21, 22). This cord of somewhat darker staining cells seems to form only in epidermis that is several layers thick. It extends almost horizontally above the basal layer and very gradually reaches the higher layers and the periderm (Fig 33). Very soon keratohyalin granules appear in the central cells, sometimes first near the superficial posterior end of the structure, sometimes deeper down A little later the central cells become quite eosinophilic with pyknotic nuclei and form a keratinized core surrounded by a tubular layer of granular cells and one or two outer layers of prickle cells. In cross section (Figs 7, 35, Plate III, 15) this structure, the hair canal, looks very much like the keratinized intraepidermal portion of the eccrine sweat duct of adult skin (it lacks, however, the PAS-resistant cuticle of the duct).

The hair canal may be relatively short (Figs. 34, 37) or very long (Fig. 36). If the follicle extends down into the corium rather steeply, *there is a sharp kink between it and the canal (Figs. 21, 33). If, however*, the follicle slants as it usually does in the Negro fetus its approach to the epidermis is almost tangential and the hair canal obviously is just the extension of the infundibulum into and through the epidermis (Figs 31, 36) The old authors wrote that the epidermis forms the hair canal in its substance. It is preferable to say that the follicle, from its origin in the basal layer, grows upward through the epidermis just as it grows downward through the corium. We shall see later how the hair canal, a purely fetal differentiation, eventually disappears. Its lower part, however, remains and becomes incorporated in the infundibulum of adult skin The pilosebaceous apparatus, throughout life, ends at the surface of the skin and not at the base of the epidermis. There is an intraepidermal "infundibular unit" (Pinkus and Steele, 1955) just as there is an "epidermal eccrine sweat duct unit" (Lobitz *et al*, 1954)

Fig 33 Fetus 3 Long solid cord of hair canal cells in epidermis Hematoxylin and eosin Magnification × 280

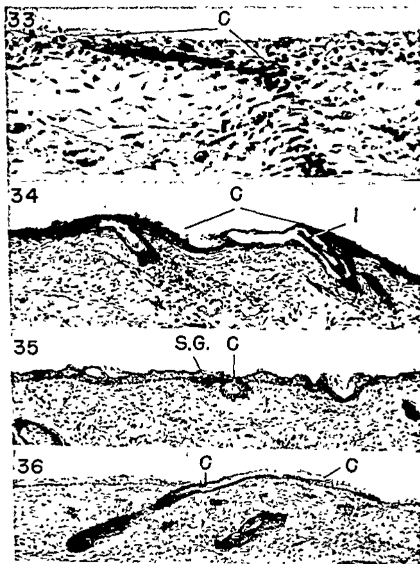
Fig 34 Fetus 4 Angle between infundibulum and hair canal. Hair and dark (basophilic) root sheath in lumen of canal, distal end of canal blocked by light (acidophilic) whorled keratin Toluidine blue Magnification × 135

Fig 35 Fetus 7, dorsal skin Cross sections of late hair canals Some are open, some plugged Note keratinization of roof of canal farthest to right Hematoxylin and eosin Magnification × 135

Fig 36 Fetus 7, pectoral skin. Very long hair canal Hematoxylin and eosin Magnification × 90

For key to lettering see legends to Figs 1–7

The unity of the subepidermal and intraepidermal portions of the hair follicle is particularly evident in PAS preparations. The cells lining the infundibulum and the hair canal at first contain much less glycogen than the epidermis, evenly scattered through all the living cells from the basement membrane in. The epidermis, on the other hand, at first is so packed with glycogen that few details are apparent. Later the basal



layer loses its glycogen completely while the upper strata retain it until fully keratinized (Fig. 25). At the acute angle between the anterior wall of the follicle and the epidermis there is a cell-sharp boundary between the two structures, with glycogen-free epidermal basal cells usually extending 2–3 cell widths down on the outer surface of the follicle (Fig. 29). The cells of the hair canal are sharply outlined as adnexal elements within the epidermis.

7. *The Hair and Inner Root Sheath*

The *hair* and its *inner root sheath* are formed by the matrix at the lower extremity of the follicle. We have seen that the matrix consists of a mass of undifferentiated small epithelial cells on the circumference of the egg-shaped mesodermal papilla. The cells around the lower half of the papilla undergo frequent mitotic division. Their products ascend higher and through mechanisms unknown are differentially converted into five dissimilar end products The first cells to show differentiation are those adjoining the inner surface of the glycogen-rich outer root sheath This one tubular layer of cells forms trichohyalin granules while still within the bulbous part of the follicle and soon after becomes keratinized (Plate III, 17) This is Henle's layer. It is a perforated thin tubular sheath consisting of large elongated squamae arranged to resemble wicker work. It is generally assumed that it forms a rigid constricting net that helps to mold the still soft inner structures.

Another tube several cell layers thick begins to form trichohyalin and keratinizes a little higher up than Henle's layer This much thicker sheath is Huxley's layer. It is coated on its inner surface by yet another

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FIG 37 Fetus 4 Tip of internal root sheath in hair canal, the distal end of which is blocked by dense keratin Hematoxylin and eosin Magnification × 135

FIG 38 Fetus 7, skin of chest Sudden fading of basophilic dark stain of internal root sheath in region of sebaceous gland Acid orcein and Giemsa Magnification × 101 25

FIG 39 Fetus 9, scalp Fading of internal root sheath in distal part of isthmus Free melanin granules in infundibulum Toluidine blue Magnification. × 168 75

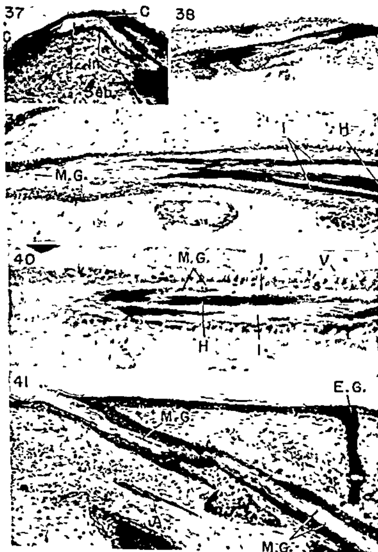
FIG 40 Fetus 9, scalp Scattered clumps of melanin in Huxley's layer contrast with dense fine granulation of hair shaft *Huxley's layer incompletely keratinized* at right end of picture, becoming increasingly basophilic toward left Toluidine blue. Magnification × 277 5

FIG 41 Fetus 9, scalp Melanin sludge in infundibulum, few granules in intact Huxley's layer in isthmus An eccrine gland is seen descending perpendicularly from the epidermis Hematoxylin and eosin Magnification· × 135

For key to lettering see legends to Figs 1–7

thin tube of shinglelike squamae, the cuticle of the inner root sheath. The inner sheath thus consists of three distinct layers

Centrally placed in this system of tubes is the hair which consists of an outer cuticle and the main body called the cortex. Fetal lanugo has no medulla The tip of the forming hair is extremely fine and always free of pigment. It is often difficult to see in paraffin sections unless stained with picric acid and is best recognized by its refractility. When fully



developed, the hair shaft is a solid cylindrical structure containing varying amounts of pigment (Figs. 29, 30). Its diameter remains relatively small and often is not larger than the thickness of the root sheath wall. The diameter of the cross section of the entire mass formed by the matrix therefore is about three times the diameter of the lanugo hair (Fig. 42).

Pigment is present in the hair as very small brown granules disposed in longitudinal rows (Fig. 29). It is derived from the dendritic melano-

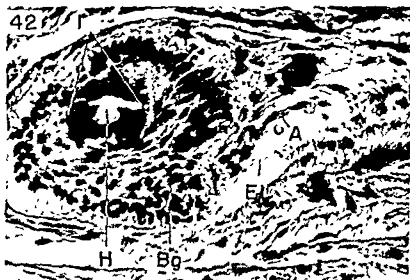


FIG 42 Fetus 10, dorsal skin Elastic fibers in the bulge area Acid orcein and Giemsa Magnification × 465
For key to lettering see legends to Figs 1–7

cytes in the matrix Contrary to what is true in adult life the lanugo hair has no monopoly on melanin Huxley's layer, in the Negro fetus, practically always contains some pigment, disposed irregularly in clumps of varying sizes (Fig 40). Another fetal characteristic is the presence of occasional flakes and clumps of PAS-positive material in the inner root sheath, most commonly found in the cuticle (Figs. 24, 29)

It is generally stated that the inner root sheath is formed as a pointed cone and that its tip pushes through the solid epithelium of the lower and upper segments of the follicle as the hard tip of a sprouting tulip bulb pierces the inert earth This description is based on the conditions of hair replacement later in life In embryonic skin, the first cells of the inner sheath form when the follicle is very short It is more appropriate to say that the matrix moves away from the tip of the root sheath and

burrows deeper, rather than that the tip pierces the solid follicle. The relatively short distance which the root sheath has to traverse before it reaches the surface is marked out by the central cells of the follicular peg which are arranged longitudinally at an early age and seem to undergo some form of degeneration or partial keratinization. Actually, one sees sometimes a disarrangement of central nuclei resembling a log jam in the isthmus, while a little higher up the lipidizing cells of the sebaceous gland begin to leave a preformed lumen Still higher up, the hair sheath meets the already keratinized portion of infundibulum and hair canal. While some canals have a central lumen when viewed in cross section (Fig. 35), others contain a solid plug of whorled keratin. Favorable sections show the tip of the root sheath resting against this plug (Fig. 37). Paradoxically, therefore, the so-called hair canal seems to offer a more solid obstruction to the advancing hair than the lower segments At this time, however, the wall of the canal and particularly *its roof become more and more keratinized* (Fig. 34). It seems likely that by continuous outward growth the distal plugged end of the canal is gradually shed, and eventually its roof disintegrates setting the hair free (Fig 41)

While in this first push the tip of the hair is sheathed by the fully stainable inner root sheath, later these coverings are not present above the level of the sebaceous gland Montagna (1956, p 190) suggests that the disappearance could be caused by chemical changes culminating in reabsorption or dissipation of the inner sheath and that this could be brought about by an enzyme (keratinase) possibly contained in the outer sheath Observations on fetal Negro skin lend some support to this thesis The fully keratinized inner root sheath, and especially Huxley's layer, stain deep blue with Giemsa solution or toluidine blue (Figs 38, 39). This basophilia is lost rather abruptly in the isthmus, but the sheath is still compact. Then, near the sebaceous gland opening, it suddenly disappears, leaving perhaps a few still basophilic flakes (Fig. 39) As was pointed out, Huxley's layer contains scattered clumps of melanin in Negro fetal hair. The space around the nude hair shaft in the infundibulum contains a much larger number of free pigment grains, often mixed with PAS-positive debris (Figs 32, 41). The impression is gained that pigment and polysaccharides remain as a sludge after the keratinous sheath in which they had been embedded has been dissolved away.

8 *The Arrector Muscle*

The last regular component of the pilosebaceous follicle is the *musculus arrector pili* Stohr (Plate I, 5, 7) points out the mesodermal nuclei between the rudiments of the bulge and the sebaceous gland as the

first anlage of the muscle. The first trace of the arrector muscle is seen in an area of increased metachromasia in the undifferentiated mesenchyme some distance from the follicle and usually at the level of the sebaceous gland. Nuclei of mesodermal cells begin to arrange themselves in chains and acquire more distinct elongated bodies. The thin cord of cells extends upward toward the epidermis and downward almost parallel with the follicle (Figs. 4, 31, 41). It usually reaches the tip of the bulge, but sometimes streaks past it and approaches the follicular wall farther down (Fig. 32). The muscle cells connect with the early collagenous fibers of the mesodermal root sheath. Elastic fibers, which later in life seem to form a tendon at both ends of the muscle, do not develop until the entire corium acquires them (Fig 42). The region around the muscle remains the most metachromatic area in the corium for a long time and also contains considerable amounts of PAS-positive ground substance. There is, however, no glycogen in the cytoplasm of the muscle fibers until a relatively late stage in development (Fig. 30) Striated muscle below the skin contains glycogen in much younger embryos

9 *The Apocrine Gland*

Many animal species have apocrine glands associated with the pilary complex In adult man, only a few specialized regions possess apocrine glands regularly, in other regions they occur occasionally Face and scalp are the most common sites of such "ectopic" glands. Although it has been stated that the embryonic human hair follicle always shows an apocrine gland anlage and that this later regresses, apocrine gland rudiments were found in only 3 out of 10 fetuses and only on a minority of the follicles of the scalp and not in the other two regions (chest and back) While this does not rule out that apocrine glands are a regular constituent of the human fetal pilary complex, the evidence rather suggests that rudiments of demonstrable size develop in some regions only and develop in "ectopic" regions on some follicles but not on all

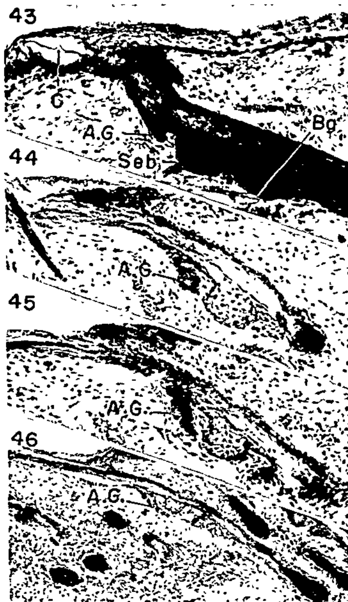
FIG 43 Fetus 4, scalp Buds of apocrine gland, sebaceous gland, and bulge on posterior wall of follicle The spur in the angle between anterior wall and epidermis probably is a secondary follicle Hematoxylin and eosin Magnification × 180

FIGS 44, 45 Fetus 5, scalp Two follicles showing more developed apocrine gland buds Several other follicles nearby showed no evidence of an apocrine gland Hematoxylin and eosin Magnification × 180

FIG 46 Fetus 5, scalp Longest apocrine gland rudiment found in this series Hematoxylin and eosin Magnification × 90

For key to lettering see legends to Figs 1–7

The apocrine gland bud appears on the posterior surface of the follicular infundibulum above the sebaceous gland. It becomes visible as a small round knob of cells and grows into a solid cord with a pointed tip and a more or less irregular shape (Figs 43–45) quite different from



secondary hair germs with which it possibly might be confused The longest rudiment observed (Fig. 46) curves around the sebaceous gland and ends in the corium above the bulge.

IV. Topography

Hairs are arranged in patterns, keeping relatively constant distances from their neighbors, and have a uniform regional slant. They usually are arranged in groups of three in man and in many other species. The laws governing the development of a limited number of follicles at regular distances probably are similar to those regulating other periodicities in embryogenesis (field effects, etc.).

In a recent detailed analysis based on examination of stained spreads of fetal human epidermis, Fleischhauer (1953b) concluded that the first hairs develop randomly over the surface, but at relatively fixed intervals (Figs 47, 48) Distribution curves show median values of 274 to 350 μ for different regions. With continued growth of the skin the first germs become more widely separated, and new anlagen develop between them when a critical distance has been reached (Fig 49). The first hairs develop more densely in some regions than in others, and regions with closely set primary germs also have smaller critical distances for new anlagen. In a fetus of 12 cm CR length hair groups begin to form, and the development of independent germs gradually subsides (Fig 50). In the common three-hair group all hairs are arranged on a straight short line more or less transverse to the direction of hair slant (Figs 20, 50). If only one secondary hair develops, this may be lateral or anterior to the first hair (Fig 22, Plate III, 14).

In order to explain specific details of distribution the phylogenetic origin of mammalian hair commonly has been invoked Many hypotheses have been advanced and hairs have been considered homologous to reptilian scales or to certain hairlike structures and sensory receptor organs of lower forms The most plausible interpretation assumes that

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Figs 47–50 Spreads of skin sliced off the preserved fetus with a razor blade and stained *in toto* Magnifications × 21

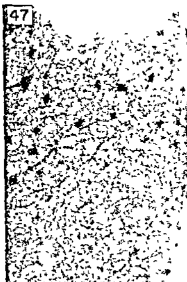
Fig 47 Fetus 1, skin of thigh, stained with hematoxylin Seemingly random distribution of evenly spaced very early round hair germs

Fig 48 Fetus 1, skin of arm, stained with hematoxylin. Slightly more advanced germs with a few very early ones in between

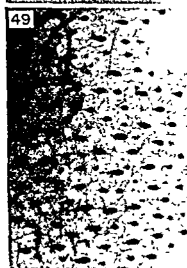
Fig 49 Fetus 3, skin of arm, stained with hematoxylin Hair stream indicated by bilateral symmetry of follicles Early primary germs in between

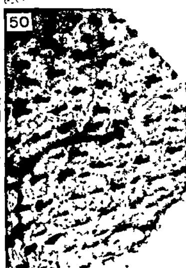
Fig 50. Slide of Dr. A A Zimmermann (cf Fig 20) stained with silver Mostly three-hair groups, some two-hair groups and some earlier individual germs

hairs develop behind the free edge of a scale, without specifying whether this is the reptilian scale or an independent analog The tail of rodents is one example where this arrangement is obvious. The assumption that development behind a scale is a general law explains at once three characteristics of the topography of pilary complexes: the slant of the hair; the disposition of the three members of a group in a straight line perpendicular to the slant; and the quincunxlike distribution of the









groups themselves. Even the ringlike arrangement of eccrine glands around hair groups may be explained on the same basis F. Pinkus (1927) introduced the term "Haarbezirk" (hair district) for the sum total of all the structures routinely associated in a compound and called attention to a nervous end organ, the "Haarscheibe" (hair disc) which if present also occupies a fixed position behind the three-hair group

Fleischhauer (1953a) has recently shown that the regional slant of the hairs, the hair stream, is foreshadowed in the bilateral symmetry of epidermal nuclei, which are almond-shaped with three diameters of unequal length and are arranged in streams long before the hair germs develop. This finding of course just pushes the unsolved question one step back, but makes it perhaps easier to explain hair streams and hair slant through general growth processes in the ectoderm.

That hair slant and general development of the follicle are intimately associated, and that the concept of a posterior and anterior side of the *pilary complex is more than a play with words*, can be seen at points where hair streams meet, as illustrated in Fig. 5. In this section the slant of hairs reverses itself, they point to the left in the left half, to the right in the right half of the picture. Simultaneously, the site of sebaceous gland and bulge is changed, remaining on the posterior side of the follicles

A variation of the theme occurs in Negro skin The follicle during its growth downward sooner or later curves so that its posterior wall becomes concave and the bulb in extreme cases occupies a position directly underneath the follicular opening (Fig 31). The entrance into the papilla, however, remains directed down toward the subcutis, and thus a sharp kink develops at the junction of bulb and lower follicle

V Summary

The morphogenesis of human hair has been delineated in close conformity with the descriptions of the old anatomists A few points that were clarified by personal investigation or seem new in fact or interpretation are the following

1 The hair germ is a differentiation product of the basal layer While there is probably a minor invagination of cellular material in the very beginning, the follicle develops mainly by mitotic division of the germ's own cells. It cannot be considered an invagination of the entire epidermis and its layers are not homologous to those of the epidermis

2 The pilary complex comprises ectodermal and mesodermal components almost from the beginning and develops through close coordination and mutual influence of either one on the other The hair follicle is a fibroepithelial structure

3 The hair canal is a continuation of the hair germ upward into the epidermis and is not part of the epidermis. In consequence, the adult follicle has an intraepidermal component, which may be called the infundibular unit, and ends only at the skin surface, not at the bottom of the epidermis. This fact is of considerable importance in the interpretation of pathological processes such as epidermal carcinogenesis (especially keratosis senilis) and wound healing.

4. The fetal hair follicle, at least that of Negro skin, contains functioning melanocytes not only in the matrix of the hair, but also in the matrix of the inner root sheath and in the basal layer of the outer root sheath. The inner root sheath contains melanin as small masses of irregular distribution.

5. The inner root sheath covers the tip of the hair during its ascent into the hair canal, but later disappears through disintegration of its keratinous components in the infundibulum Thus, in Negro skin, a *sludge of clumps of melanin and PAS-positive material is left behind* in the infundibulum

6 The mesodermal papilla of the fetal lanugo, in contrast to that of later generations does not contain a blood capillary. It is not metachromatic until the hair is fully developed.

7. Staining for glycogen reveals ebbs and tides of this substance in the various segments of the follicle The early hair germ cells loose glycogen quickly. The matrix and the hair and inner root sheath are always practically free of glycogen The outer root sheath and the bulge reacquire glycogen soon, but the sebaceous gland remains free until lipidization sets in Then the peripheral cells show granular deposits. While the epidermal basal cells contain glycogen only in the young fetus, and not later, the epithelium of the outer root sheath contains glycogen through all its layers, and especially in the tall basal cells of the lower segment. The arrector muscle acquires it relatively late

8. The vitreous membrane and specifically arranged collagenous fibers of the mesodermal root sheath develop early, but elastic fibers here and at the insertion of the arrector muscle differentiate only contemporaneously with those of the remainder of the corium

9 The musculus arrector pili develops through the alignment of mesenchymal cells at some distance from the follicular wall in a region of early and pronounced metachromasia of the ground substance. It becomes secondarily connected with the fibrous root sheath in the region of the bulge

10 Apocrine gland rudiments of visible dimensions develop on a minority of follicles on the scalp They were not observed on chest or back They probably are not a constant part of all human hair follicles

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CHAPTER 2

The Regional Frequency and Distribution of Hair Follicles in Human Skin

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I INTRODUCTION

The methods used by Schultz (1931), Miner (1951), and Thomson (1954) in studying the distribution of hair follicles and sweat glands in human skin yielded interesting results, but are not complete. In this investigation the total number of hair follicles and sweat glands was estimated by counting them in whole mounts of epidermal preparations, obtained by the "skin splitting" technique (Medawar, 1941; Szabó, 1955) Since all specimens were treated in exactly the same way, no attempt was made to correct these figures to correspond to those in unfixed, normal skin, and the results are valid for comparative purposes

All types of hair follicles, coarse and fine, and in all stages of the hair growth cycle were counted The figures, therefore, include all follicles, and not just those of macroscopically visible hairs. The averages given in the following tables are estimated for 1 cm² and the standard error of the mean is also given In all the tables, counts for the hand and foot are excluded from the averages of the extremities.

II. RESULTS

Table I shows that hair follicles are most numerous on the cheek and forehead and their relative proportion to sweat glands is also the most favorable for them Scalp is not included since good specimens were too few to give significant data

The standard error is very high in Table I, due to the great individual variation and to the relatively small field which could be counted by this method Table II however, which gives the summarized averages for these appendages, shows a smaller standard error

There are about 4 to 6 times as many appendages per cm² in the cheek and forehead than in the trunk of extremities Although the in-

dividual variation is large, there is no significant sexual variation in the distribution of hair follicles on the face (Table III).

TABLE I

THE REGIONAL FREQUENCY DISTRIBUTION OF HAIR FOLLICLES AND SWEAT GLANDS IN THE ADULT SKIN

Area	Number of specimens investigated	Number of hair follicles ± S E mean	Number of sweat glands ± S E mean	Proportion sweat glands. hair follicles
Cheek	11	880 ± 60	320 ± 60	0 36
Forehead	4	770 ± 60	360 ± 50	0 48
Forearm	*5*	*100 ± 50*	*220 ± 30*	*2 2*
Thigh	21	55 ± 5	125 ± 5	2 3
Leg	5	50 ± 20	150 ± 20	3 0
Upper arm	10	40 ± 10	140 ± 20	3 5
Abdomen	3	(40 ± 30)	210 ± 20	(5 2)

TABLE II

REGIONAL FREQUENCY DISTRIBUTION OF SKIN APPENDAGES IN THE ADULT

Area	Number of specimens investigated	Number of appendages ± S E mean
Forehead	4	1120 ± 70
Cheek	*11*	*1050 ± 80*
Forearm	5	320 ± 30
Abdomen	4	280 ± 20
Chest	4	250 ± 20
Leg	5	200 ± 20
Thigh	21	180 ± 10
Upper arm	10	180 ± 20

TABLE III

SEXUAL VARIATION IN THE DISTRIBUTION OF HAIR FOLLICLES IN THE CHEEK

	Number of specimens	Average
Male	4	770 ± 160
Female	4	730 ± 130

For the study of the development of this great regional variation the summary in Table IV gives the averages of appendages for the main body regions.

Taking the sum of the four averages for one hundred, the relative

density per unit area is 58% on the head, and 11 to 16% in the rest of the body.

Assuming that in a full-term fetus the skin appendages appear in equal numbers in all of the four main body regions, the percentage distribution would be 25-25-25-25. However, during postnatal development, the total surface increases about three times in the head and about ten times in the rest of the body (Boyd, 1935). Supposing that

TABLE IV
AVERAGE NUMBER OF SKIN APPENDAGES FOR THE MAIN BODY REGIONS

Area	Average	Relative density/unit area
Head	980 ± 10	58%
Trunk	270 ± 10	16%
Upper extremities	250 ± 10	15%
Lower extremities	190 ± 10	11%

TABLE V
THE DILUTION OF AN ORIGINALLY EVENLY DISTRIBUTED POPULATION OF SKIN APPENDAGES DURING POSTNATAL DEVELOPMENT

Area	Full term fetus appendages	%	Rate of growth	Adult appendages	%
Head	1000	25	x 2 98	335	52
Trunk	1000	25	x 9 02	111	17
Upper extremities	1000	25	x 9 55	104	16
Lower extremities	1000	25	x 10 46	95	15

the number of appendages, once established in the fetus, does not increase in later life, a corresponding threefold or tenfold "dilution" of the originally evenly distributed population of appendages would then take place (Table V)

The hypothetical percentage figures of 52-17-16-15 are very close to those in Table IV: 58-16-15-11

A percentage distribution has also been calculated assuming that the number of appendages is established between the third and ninth fetal month Table VI gives these figures, showing that the actual distribution of appendages in the adult fits between the fifth and sixth fetal month.

Tables VII and VIII show the actual number of appendages found in the fetus.

Table VII shows that the rapid decrease in the number of appendages in the thigh corresponds to the calculated tenfold decrease,

TABLE VI
VARIATION IN THE PERCENTAGE DISTRIBUTION OF SKIN APPENDAGES PER UNIT AREA IN THE ADULT SKIN, ACCORDING TO THEIR APPEARANCE IN FETAL LIFE

	Months *in utero*							
Area	3	4	5	!ᵃ	6	7	8	9
Head	68	62	59	58	56	54	53	52
Trunk	16	17	17	16	17	17	17	17
Upper extremities	9	12	14	15	16	16	16	16
Lower extremities	7	9	10	11	12	13	14	15

[a] ! = actual variation found in adult (Table IV).

TABLE VII
AGE CHANGES IN THE REGIONAL FREQUENCY DISTRIBUTION OF SKIN APPENDAGES IN FETAL SKIN

	24 weeks	7 months	Full term	Average for adult
Cheek	4780 ± 110	3420 ± 40	1670 ± 70	1050 ± 80
Thigh	3980 ± 780	2300 ± 70	2040 ± 300	180 ± 20

TABLE VIII
AGE CHANGES IN THE FREQUENCY DISTRIBUTION OF SKIN APPENDAGES IN THE THIGH

Baby[a]	Adult	Senile
710 ± 60	180 ± 10	150 ± 20

[a] Average of four estimates, each under 2 years old

and on the cheek (with the exception of the yet unexplained data on the full-term fetus) to the threefold decrease. It is also shown in Table VIII that there is a rapid decrease in the density of appendages in the thigh during postnatal development, whereas the difference between the averages for the adult and senile skin are not significantly different

Finally, Table IX shows the total number of appendages in the four main body regions.

This table shows that although the regional variation in the frequency distribution of appendages per unit area is high, their absolute numbers do not differ much between the head and the rest of the body.

TABLE IX
TOTAL NUMBER OF SKIN APPENDAGES IN THE MAIN BODY REGIONS, CALCULATED FOR THE WHOLE SURFACE AREA OF A 24-YEAR-OLD ADULT

Area	Total number	%
Head	1,087,300	22 4
Trunk	1,640,250	33 7
Lower extremities	1,282,500	26 4
Upper extremities	850,000	17 5

III. COMMENT

There is a correlation between the differential rate of growth of the body surface and the regional frequency distribution of skin appendages. Consequently, it is assumed that sweat glands and hair follicles do not form *de novo* in adult skin, and that their absolute number does not increase This, however, does not exclude the possibility of the neogenesis of hair follicles under experimental conditions of wound healing (Breedis, 1954, Billingham and Russell, 1956).

There is no large scale destruction of hair follicles during postnatal development, as it was sometimes assumed, but there is a dilution of an originally dense population of appendages during the growth of the body surface The relative proportion of sweat ducts to hair follicles varies greatly from region to region The number of epidermal downgrowths appear in similar numbers in all of the main body regions, they, however, are met by a high number of dermal papillae only in the head, giving rise to a dense population of hair follicles The difference in the regional frequency distribution of dermal hair papillae, it seems, is very high even in the earliest stages of fetal development. The relatively low number of such papillae, combined with a high rate of expansion of the body surface in the trunk and extremities is the reason for a low density of hair follicles

Skin appendages do not appear simultaneously everywhere. The eyelashes and hairs on the face develop in the third to fourth fetal months and the hair growth in the trunk and extremities follows later. The time given in Table VI (fifth to sixth months) is only a mean time between the development of the hairs on the head and the rest of the body

These observations show that the number of hair follicles of the cheek do not differ significantly in men and women. Secondary sexual difference in the piliary system of the face, therefore, must be physiological.

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CHAPTER 3

The Anatomy of the Hair Follicle

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I Introduction

Within biological systems, the hair follicle offers a relatively simple model for studying growth and differentiation A follicle of the human scalp, for example, produces approximately 0 35 mm. of hair shaft per day (Myers and Hamilton, 1951) Regardless of its size, only a small mass of cells at the base of the follicle, the matrix, produces the hair. The daily metabolic requirements to carry on mitotic activity by this germinative tissue, and for the synthesis of complex proteins farther up in the follicle, to produce this much hair is of a magnitude greater than is found in most other tissues. Within each follicle, mitotic activity of the cells of the matrix and synthesis of protein go on unceasingly as long as the hair is growing, growth, however, is periodically abruptly arrested, and generation of a hair ceases. At this time there is a destruction of the major portion of the hair root, and what cells remain enter a period of absolute quiescence. After a variable interval of time the dormant follicle bursts into activity, a period of organogenesis follows, during which an entirely new hair root is regenerated and the production of a hair is resumed

Growth, regulation of growth, cellular death and destruction, and

tissue regeneration are perhaps not so clearly involved in the normal physiologic behavior of other biologic systems as they are in the hair follicle. Many of the cytological and histological changes associated with these phenomena have been described, but the mechanisms that control growth and differentiation are largely unknown To understand some of the biological principles that regulate hair growth, it is necessary to keep in mind the anatomy of the hair and its follicle. The descriptions that follow deal primarily with human hair follicles, and although differences occur, the basic pattern of the follicles in other mammals is the same. For a more extensive treatment of the histology and cytology, the reader is referred to "The Structure and Function of Skin" (Montagna, 1956).

II Gross Anatomy

A. *The Hair Follicle*

Those who are familiar only with the occasional fragment of hair follicles, cut at various levels in histological sections of skin, have little concept of their three-dimensional aspect The relations of the various structures, the differences in the sizes of different follicles and at different states of activity, and the general groupings of follicles, can only be appreciated in models reconstructed from complete serial sections This tedious and painstaking work is mostly out of favor in an era concerned with ultrastructure, but there is no substitute for it and the results are gratifying The series of photographs which follows has been chosen from a large number of models reconstructed in balsa wood (Figs 1–6) Each of these figures shows the reconstructed model on the left, arrows from certain levels of the models point to photomicrographs on the right, representative of that particular level of the follicle In all cases the sebaceous glands are represented in white These reconstructions were made at the identical magnification, and the differences in size are real

Figure 1 is a cluster of three pilosebaceous units from the scalp The follicle on the left of the group, much shorter than the one on the right, is quiescent and contains a club hair, marked *C* in the histological section The large follicle on the right is growing, and the lower of the three photomicrographs shows its proliferative matrix, with the dermal papilla in the center. The middle arrow is from the level of the keratogenous zone, in the middle photomicrograph, *I*, indicates the inner root sheath, *E*, the outer root sheath The middle unit of this cluster contains no hair, which was probably lost through a degeneration, and is now a duct for the sebaceous gland All three of these units share a common orifice at the surface Figure 2 is a model of two follicles from the upper

back, sharing the same pilary canal The small follicle on the left, seen just under the sebaceous gland, is quiescent, the one on the right is growing. *Figure* 3 is a group of four follicles from the scalp sharing one canal; the follicle on the extreme left, obscured by the sebaceous glands, and much shorter than the others, is quiescent; the other three follicles are growing. This shows clearly the difference in the size of growing

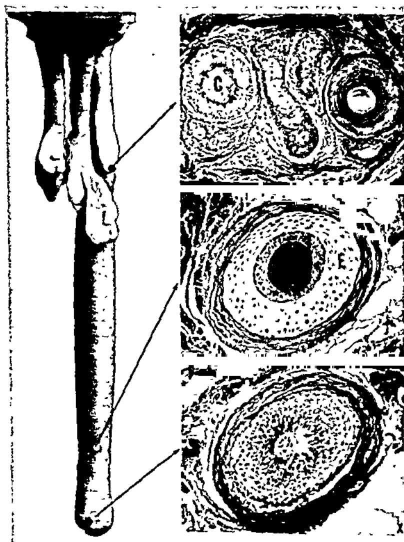


Fig 1 Balsa wood reconstruction of a cluster of three pilosebaceous units from the scalp of an adult man

and resting follicles. The follicle at the right has no sebaceous gland, but this is not rare In Fig. 4, a reconstruction of a group of follicles from the upper back, two follicles come together just above the entrance

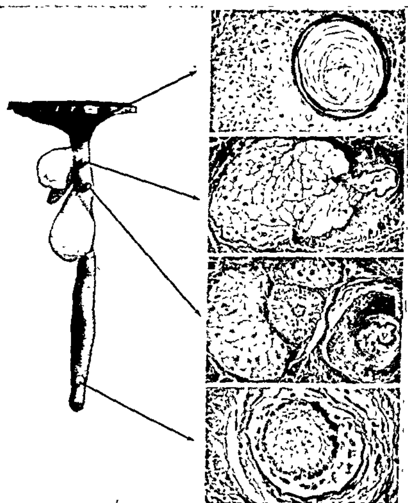


FIG 2 A pilosebaceous unit from the upper back of an adult man

of their separate sebaceous glands and they share the same canal, indicated by the middle arrow These relatively short follicles are both quiescent. Several vellus hair follicles are found on each side of the principal follicle Figure 5 shows two growing follicles from the upper back of a child These are much smaller than the follicles of adults, and the sebaceous glands are diminutive, compare these with the other

models, which are all from adult skin Finally, Fig. 6 is a follicle of a beard hair of a man. Beard hairs are thick and have relatively enormous follicles, which have some distinctive features. The upper photomicrograph shows the orifice of the pilary canal, divided into two distinct channels separated from each other by keratinous material. The hair

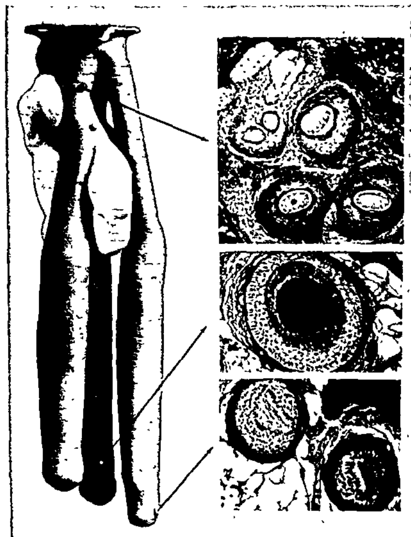


FIG 3 Group of four pilosebaceous units from the scalp of an adult man. The quiescent follicle on the extreme right is barely visible under the sebaceous gland

FIG 4. Cluster of pilosebaceous units from the upper back of an adult man

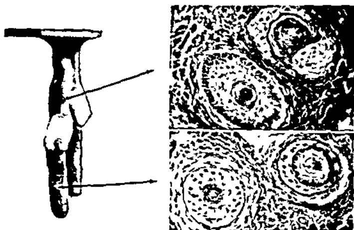


FIG. 5. Two pilosebaceous units from the back of a girl 7 years old Compare with Figs 1 and 3

passes through one channel, and the sebum from the associated sebaceous gland passes through the other. This is a peculiarity of the pilosebaceous units of the beard. The lower three photomicrographs show the changes in the contours of the hair and outer root sheath at different levels of the follicle. Several vellus hair follicles surround the main pilary unit.

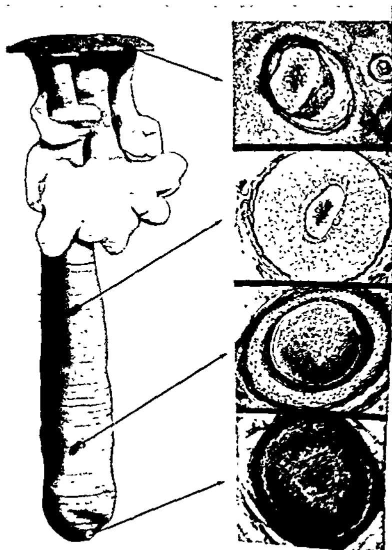


FIG. 6 Pilosebaceous unit of the beard of an adult man.

B. *The Hair*

Hair is the keratinized gross fiber produced by the hair follicle. Its morphologic characteristics vary from one species of mammal to another. Even within the same species, the hairs from one region of the body may differ markedly from those from another region: some are stiff, some intermediate, and others soft and woolly. Even within a single region, *hairs of different length and texture are characteristically found* The longer and coarser ones are the terminal hairs, the shorter, soft



Fig 7 A Surface of the scalp showing hairs emerging singly or in groups B Oval hair, twisted along its axis C Round hair

ones, vellus hairs, constitute the underfur in other mammals A sharp classification of hairs into these two types cannot be made on the basis of length alone. For example, the hairs of the scalp are characteristically longer than those of the back, but terminal and vellus hairs are present in both regions, and the length of terminal hairs of the back may approximate that of vellus hairs of the scalp The length of hairs in each region is intermediate between that of the "terminal" and "vellus" hairs of that region

Hairs show extreme variations in color, diameter, and transverse contour. Some hairs may be almost perfectly round, while others are so markedly flattened or oval that they resemble ribbons Twisting of

such ribbonlike hairs along the longitudinal axis may give the impression that the hair varies widely in diameter (Fig. 7), although this is not the case, except in certain pathological conditions.

Hairs emerge from the skin either singly or in groups of two or more (Fig. 7). Two hairs may arise from a single pilosebaceous unit, as is frequently the case on the back of man. The emergence of several hairs from a single follicular orifice, however, does not indicate the presence of a compound type follicle, in the scalp several individual pilosebaceous units frequently converge near the surface and share a common follicular opening (Figs. 1–6).

III Microscopic Anatomy

A. *The Hair Follicle*

Hair follicles are fairly simple organs which consist primarily of sleeves of epithelium continuous with the surface epidermis. Follicles grow slanted into the dermis, and the base of the longer ones extends below the level of the dermis into the panniculus adiposus. The follicle attains its greatest diameter at the base, where it is dilated into a clavate bulb Inside of the bulb is an egg-shaped cavity that is completely filled with loose connective tissue, the dermal papilla The arrectores pilorum muscles are attached to the bulge of the follicle, on the side of the follicle that forms the obtuse angle to the surface, and extend to the surface of the dermis (Fig 8). Clusters of sebaceous glands are couched above the muscles and enter the upper part of the follicle through a duct of varying length. Some follicles have more than one sebaceous gland growing from them

In the center of the follicle is the hair, whose root extends down to the bulb Around the hair is the inner root sheath, and on the outside is the outer root sheath (Fig 9). Hairs have a medulla of variable thickness, which is often discontinuous or absent, a thick cortex, which forms the bulk of the hair, and a one-layered cuticle on the outside. The inner root sheath is composed of an inner layer one cell thick, the cuticle, a middle Huxley's layer one or two cells in thickness, and an outer Henle's layer one cell thick The cuticle cells of the inner sheath are flattened and shingled, and their free edges are directed downward, interlocked with the cuticle cells of the hair, which are directed upward. Outside the outer sheath is a hyaline "glassy" membrane. The entire follicle is surrounded by a connective tissue sheath, composed of an inner layer with fibers oriented horizontally, and a thicker outer layer with fibers oriented vertically The connective tissue sheath is actually a continuation of the papillary body of the dermis and is attached to the dermal papilla at the base of the follicle In this way, the hair follicle is not

in contact with the fibrous reticular layer of the dermis, but is separated from it by the connective tissue sheath.

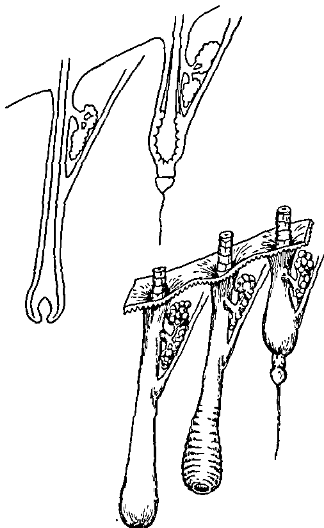


Fig 8 *The diagram on the upper left shows a growing follicle, the one on the upper right a quiescent one* The lower diagram shows a follicle in anagen on the left, one in catagen in the middle and one in telogen on the right

When follicles cease to produce a hair, they shrivel up and the lower part, or bulb, largely degenerates Quiescent follicles are simpler and shorter structures than active ones At the base of a resting follicle, the hair forms a club that is anchored by thin keratinous strands to the epi-

thelial sac around it. The club is surrounded by a hyaline capsule, or vestige of the inner root sheath, this continues up to just below the duct of the sebaceous gland, where it becomes wrinkled and fragmented. Around the capsule, the remaining outer root sheath forms a thick epithelial sac, at the base of which is a peg of cells (Fig. 10); at the

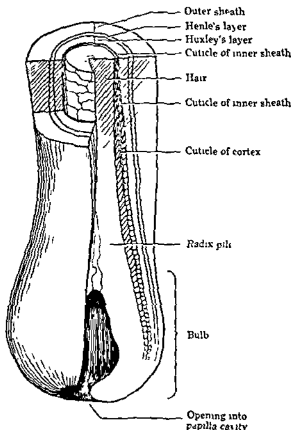


FIG 9 Stereogram of the lower part of an active follicle

flattened base of the peg is the ball of dermal papilla cells, no longer incapsulated by a bulb. This *pedicle of cells* and the lower part of the epithelial sac comprise the hair germ, from which the next hair generation develops

Growing hair follicles are said to be in *anagen*, quiescent ones in *telogen*, and the period of transition between the two, *catagen* (Dry, 1926). Whether or not one likes the terms, they are in common usage, and they are convenient.



FIG 10 Montage of a quiescent follicle from the external auditory meatus. The arrow points to the basal epithelial peg

B. *The Bulb*

The bulb of the hair follicle can be divided into a lower region of undifferentiated cells and an upper region in which the cells become differentiated to form the inner sheath and the hair (Figs. 11 and 12). A line across the widest part of the papilla would separate the two regions at the critical level (Auber, 1952). Below the critical level is

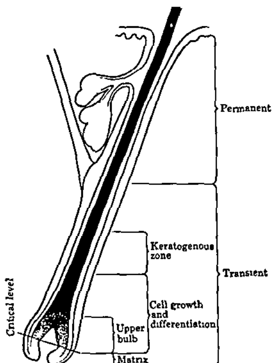


FIG 11. Diagram of an active follicle showing the various regions The arrector pili muscle and the sebaceous glands have been drawn deliberately on the wrong side so as not to interfere with the labeling

the matrix, or germination center of the follicle, where every cell is mitotically active From the matrix, cells move to the upper part of the bulb, where they increase in volume and become elongated vertically (Fig 13) Some of the cells in the upper bulb still show some mitotic activity, but these are too few to account for much of the growth of the hair.

The upper bulb can be divided into four regions Immediately above the critical level, in the wide portion of the bulb, is the pre-elongation

region, where the cells align themselves vertically and become slightly larger (Auber, 1952). Above this region, where the diameter of the bulb is narrower and the cells become conspicuously elongated, is the

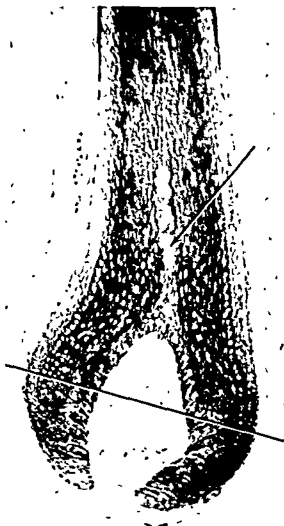


FIG 12 Lower third of a follicle from the scalp The lower line is drawn across the critical level, the upper line points to the medulla

cellular elongation region Farther up, in the cortical prekeratinization region, distinct fibrils stainable with basic dyes can be seen Immediately above this region, in the keratogenous zone (Giroud and Bulliard, 1930), the cells become hyalinized, and the keratin of the hair is

stabilized; distinct fibrils can be seen only with certain techniques and under polarized light. Depending upon the length of the follicle, the keratogenous zone terminates at approximately one-third of the way



Fig 13 Detailed photograph of the matrix, below the line, showing the cells streaming up into the upper bulb

between the tip of the papilla and the surface of the skin The mature hair above the keratogenous zone shows no distinct cellular elements, and has a narrower diameter

Melanin in the follicles is distributed in a remarkably rigid pattern. Since the matrix is nearly free of it, the separation of the upper from

the lower bulb at the critical level is very clear. The outer and inner sheaths are free of melanin. Traces of pigment may occasionally spill into the inner sheath, and very small dendritic cells are found there on rare occasions, but the dividing line is very sharp between the cells of the hair proper, which are pigmented, and those of the sheaths, which are not.

Most of the tall columnar cells lining the dermal papilla are melanin-producing, dendritic melanocytes. The dendrites that radiate from these cells are insinuated between the undifferentiated cells of the cortex and the medulla These melanocytes are larger, but otherwise similar to those found in the epidermis. As the cells of the hair cortex move up from the matrix, they acquire pigment granules

The upper bulb is comparable to the spinous layer of the epidermis In both places, indifferent epidermal cells become larger, acquire pigment, synthesize fibrous proteins, become reoriented, and undergo the final steps of keratinization

C. *The Hair*

All hairs have a cuticle on the outside, and a cortex, some of them a medulla in the center. The cuticle is a single layer of imbricated scales, with the free margins directed up, they are translucent and free of pigment The cells of the cuticle of the hair are interlocked with those of the inner root sheath, firmly anchoring the hair in the follicle The cuticle binds the cortex, which without this protection would become frayed and fall apart. Cuticles are classified as coronal when each cell completely surrounds the hair, and as imbricate when they do not The free edge of cuticle cells may be simple, dentate, or serrate. Cuticle cells may be elongate, acuminate, ovate, and flattened

The mass of the hairs is formed by the cortex, which is composed of fusiform, keratinized cells cemented together; in pigmented hairs, melanin granules are aligned longitudinally in these cells. Between *the cells of the cortex are found variable numbers of delicate air spaces* called fusi (Hausman, 1932, 1944). In the living portion of the hair root the fusi are filled with fluid, as the hair grows and dries out, air replaces the fluid

The medulla may be continuous, discontinuous, or fragmental. In coarse hairs it is usually continuous or fragmental (Fig. 12); in fine hairs it is discontinuous or absent The type of medulla present can vary even within the same hair The medulla is composed of large, loosely connected keratinized cells Large intra- and intercellular air spaces in the medulla determine to a large extent the sheen and color tones of the hair by influencing the reflection of light

Changes occur in the scalp hairs from childhood to maturity (Duggins and Trotter, 1950, 1951; Trotter, 1930, 1932, Trotter and Dawson, 1931, 1932, 1934, Trotter and Duggins, 1948, 1950). The diameter of hairs increases less rapidly and uniformly during the first 3 or 4 years after birth, it increases less rapidly and less uniformly during the next 6 years; then it increases slowly or not at all Few of the hairs have a medulla at birth, the percentage of medullated hairs increases rapidly during the first 7 months. From 7 months to the second year, the percentage of medullated hairs decreases, a period of great irregularity follows and then the percentage tends to rise slightly at 5 years From birth to 2 years of age, the percentage of hairs with a medulla is slightly higher in girls than in boys, from the ages of 2 to 6, the percentages are similar, and from the ages of 6 to 14 the percentage is much higher in boys. Boys exhibit a higher percentage of scalp hairs with broken or continuous medullas than girls. Negro children have a higher percentage of medullated hairs than do white children. Usually, the number of cuticular scales per unit area drops slightly during the first year, but later there is no specific trend. Although adult human scalp hairs are believed to be rarely medullated, when viewed under polarized light, all hairs, with the exception of the very fine ones, show a fragmental or discontinuous medulla The medulla may be only one or two cells in diameter, but it is present nonetheless. Such hairs would appear nonmedullated when observed under the light microscope.

1. *The Cuticle of the Hair*

The cells of the cuticle can be recognized in the upper part of the bulb as they sweep upward as a single row from the matrix About midway in the bulb these cells are cuboidal and stippled with numerous basophilic granules. When the cells reach the upper region of the bulb, they become columnar, with the long axis oriented radially. They retain this orientation for a short distance above the bulb, and then their outer edges begin to be tipped upward as though the inner sheath lateral to them were growing faster than the hair and swept the edges upward Since these cells are at least twice as broad as they are high, they become imbricated when the outer sides shift apically. As their orientation shifts from a horizontal plane to a vertical one, the cells become flattened squamae This reorientation is completed below the midway mark of the follicle In the upper half of the follicle, these cells become hyalinized, their nuclei disappear, and the mature cuticle adheres to the cells of the cortex

The inner sheath must grow at the same rate as the hair, or faster If the cuticle cells of the inner sheath moved upward faster than those

of the hair, they could pull the lateral borders of the cuticle cells of the cortex in an upward position and reorient them; other factors, however, could bring about these movements.

The cuticle cells have different staining properties than the surrounding structures. Unlike the cells of the inner sheath, the cuticle cells of the cortex do not elaborate visible trichohyalin. With Altmann's acid fuchsin-methyl green, the cuticle cells of the cortex stain with methyl green from above the bulb up to the level of the sebaceous ducts, above which they become gradually fuchsinophil (Montagna, 1956). In these same preparations the cortex stains with methyl green only in the region just above the bulb, the cortex is fuchsinophil from the keratogenous zone upward The medulla cells stain with methyl green until they become keratinized, in the upper half of the follicle. This staining reaction indicates that the cuticle is not fully keratinized until it reaches the upper third of the follicle. The cells of the inner sheath are brilliantly fuchsinophil as soon as they acquire trichohyalin

The selective stainability with acid fuchsin and methyl green may denote chemical differences in the keratins. The keratin of the cuticle of the cortex seems to be different from that of the cuticle of the inner sheath This might explain the split that occurs between the hair and the inner sheath

D. *The Inner Root Sheath*

The inner root sheath of active hair follicles consists of Henle's and Huxley's layers and the cuticle. All three layers arise from the peripheral and central and lateral mass of cells of the matrix. The cells move upward and laterally from the matrix (Fig. 13) and become arranged into three concentric layers in the upper bulb. The cells in all three layers of the inner sheath accumulate characteristic, hyaline "trichohyalin" granules

The cells of Henle's layer acquire trichohyalin granules immediately after they have arisen from the matrix These cells are first cuboidal but become elongated vertically in the upper bulb When they first appear, granules are very small, as the cells move upward, they coalesce into large homogeneous globules and parallel rods In the upper region of the bulb the cells become completely hyalinized, their nuclei become indistinct and finally disappear. Unlike the cells in other keratinizing tissues, those of the inner sheath do not decrease appreciably in volume

In the cells of Huxley's layer, trichohyalin granules first appear at the summit of the bulb, at which point the cells of Henle's layer are completely hyalinized Some cells without trichohyalin granules send lateral cytoplasmic processes across Henle's layer, and penetrate as far as

the axial layer of the outer sheath. These so-called Flügelzellen (Hoepke, 1927) represent living bridges of cytoplasm across the dead Henle's layer. All nutrients or energy sources from the outer sheath to the hair root come across these bridges. About midway up in the follicle, the cells of Huxley's layer are hyalinized.

The cells of the cuticle of the inner sheath do not acquire trichohyalin granules until they are about halfway up in the follicle. They are the smallest cells in the follicle and can be recognized even in the lower bulb. Above the bulb, the nuclei of the cuticle cells become elongated vertically. They remain small and compressed up to nearly halfway in the follicle, where they begin to show a few small trichohyalin granules At this level the cells are somewhat flattened, and the proximal edges become slightly dislocated axially so that they overlap the distal ends of the cells below them Shortly after they acquire trichohyalin granules, the cuticle cells become hyalinized and their nuclei fade away. Above the middle of the follicle, the three layers of the inner sheath all become fused and hyalinized.

The inner sheath is eliminated in the pilosebaceous canal. The disappearance could be due to chemical changes which culminate in a dissipation of the inner sheath, by some enzyme, perhaps keratinase (Stankovic *et al*, 1929), which would digest it selectively. The hair shaft, being slightly more acidic and covered by an epicuticle, consisting of lipids and carbohydrates, would be protected from enzymatic action At the place where the inner sheath begins to be dissipated are concentrated large amounts of AS esterase (Montagna and Ellis, 1958), which may also play a role in keratinolytic activity.

Since it is interlocked with the hair, the inner sheath grows and travels outward at approximately the same rate as the hair The outer side of Henle's layer must slide over the axial border of the outer sheath, which is stationary These two layers have very smooth surfaces at the interphase, which facilitates the movement of the inner sheath. The cells of Henle's layer are keratinized immediately after they rise from the matrix and slide easily against the partially keratinized axial cells of the outer sheath

E The Outer Root Sheath

The thickness of the outer sheath is uneven, causing the hair to be more or less eccentric in the follicle Most of the follicles have some degree of swelling of the outer sheath on the side of the bulge When the bulb is bent or curved, as in the scalp of Negroes, the outer sheath is thicker on the convex side At the level of the sebaceous glands and above, the outer sheath is indistinguishable from the surface epi-

dermis; below the sebaceous glands the cells contain large amounts of glycogen, and in histological preparations appear vacuolated. The cells in the middle third of the follicle, which contain more glycogen than those elsewhere, are reduced to flimsy, spongy sacs.

The outer sheath extends all the way to the tip of the bulb, around which it is composed of two layers of greatly flattened cells. Just above the bulb the outer sheath attains three layers. It becomes gradually pluristratified and attains its greatest thickness a third of the way up the follicle. At this point nearly all of its cells are riddled with vacuoles, with the exception of those in the axial border. The peripheral cells are tall columnar and oriented perpendicular to the axis of the follicle. *In the upper third of the follicle, none of the cells of the* outer sheath are highly vacuolated and the peripheral cells are cuboidal

The peripheral columnar cells in the lower half to two-thirds of the follicle possess minute basal cytoplasmic processes which push laterally through the thickened vitreous membrane, best developed in the region just above the bulb. The peripheral cells in the upper third of the follicle have a fairly smooth base.

Over the entire length of the outer sheath, the cells at the periphery are more vacuolated than those in the axial border, which show a relatively intact cytoplasm Intercellular bridges, nodes of Bizzozero, and tonofibrils are particularly well developed in the more axially located cells. The cells that rest against Henle's layer are rich in tonofibrils, about halfway up the follicle their cytoplasm becomes hyalinized and undergo partial keratinization (Gibbs, 1938).

Some mitotic activity is encountered in the upper part of the follicle, where the outer sheath blends with the surface epidermis This part is similar to the surface epidermis and forms a keratinized surface layer which is constantly being sloughed off Lower down in the follicle the outer sheath is a morphologically static structure. Both necrotic cells *and mitotic figures are occasionally found in the outer sheath,* cell death and cell division seem to bear a direct relation to each other

Active hair follicles can be divided into upper and lower halves (Fig 11) The lower half is mostly a transient structure that comes and goes with the proliferative activity of the hair follicle. During catagen and subsequent telogen, the outer sheath in the upper half of the follicle forms at least part of the "hair germ" and the epidermal sac around the club hair.

F. The Vitreous Membrane

The vitreous membrane is a noncellular partition that separates the external root sheath from the connective tissue sheath It is thicker around the lower half of the follicle and thickest around the widest part

of the bulb; it is very thin around the lower part of the bulb and nearly impossible to demonstrate in the papilla cavity.

The membrane is composed of two layers, allegedly secreted by the cells of the outer sheath (Spuler, 1899, Stohr, 1903). Cooper (1930) believes that only the inner layer of the vitreous membrane is formed by a condensation of granules secreted by the basal cells of the external sheath; others claim, however, that the glassy membrane originates from the connective tissue sheath (Hoepke, 1927; Merkel, 1919). The outer layer of the vitreous membrane surrounds the entire follicle and is continuous with the basement membrane of the epidermis, it is composed principally of delicate longitudinally arranged collagenous fibrils. In the lower half of the follicle, around the tall columnar basal cells of the outer sheath is a second, inner layer. This is a tangled skein of fibrils wound horizontally between the cytoplasmic processes of the peripheral cells of the outer sheath. The mixture of these fibrils and the cytoplasmic processes of the basal cells gives rise to the irregular, heterogeneous inner lamina. The fibrils stain a brilliant red with the periodic acid-Schiff technique, and they stain metachromatically (Montagna, 1956). Fibrils are more prominent around the region of the outer sheath in which the basal cells are particularly rich in metachromatically staining granules. This close relation between the metachromatic granules and the fibrillar lamina suggests that these fibrils may be derived from the metachromatically staining granules (Cooper, 1930).

The skein of fibrils in the inner layer becomes extremely thick during early catagen, but the outer layer remains unchanged. The hypertrophied inner layer forms a wrinkled sac around the degenerating lower part of the follicle during catagen At telogen the hypertrophied glassy membrane around the degenerating lower half of the follicle becomes fragmented and is resorbed Resting hair follicles are surrounded only by a thin hyaline membrane which corresponds to the outer lamina.

G The Connective Tissue Sheath

The connective tissue sheath is composed of an inner layer with fibers arranged circularly, and a thicker outer layer with longitudinal fibers Both layers are composed of collagenous fibers, a few elastic fibers, and fibroblasts, neither layer abounds in cells, but more may be found in the outer one.

The blood vessels of the follicle, to be described later, are embedded in the connective tissue sheath Plexuses of capillaries are found mostly in the inner layer, the straight, parallel arterioles in the outer layer.

The morphology of the connective tissue sheath changes together with that of the follicle during activity and quiescence. In early catagen, the connective tissue sheath becomes wrinkled, and the inner layer is enormously thickened. In late catagen, when the lower half of the follicle degenerates and the base of the follicle withdraws within the dermis, the connective tissue sheath becomes excessively folded and wrinkled (Fig. 8). At the completion of catagen, quiescent follicles are less than half their former length and the connective tissue sheath around them is not crisply delineated. The thickened connective tissue sheath becomes fragmented and is resorbed, leaving only a wispy trail in the area vacated by the follicle.

The connective tissue sheath is continuous with the thin layer of areolar tissue that surrounds the sebaceous glands, and with the papillary layer of the dermis. It is greatly attenuated around the bulb, and its fibers are arranged most longitudinally. At the base of the follicle it is attached to the dermal papilla by a stalk. The connective tissue sheath, then, the papillary layer of the dermis, and the dermal papilla comprise a continuous unit of tissue

H. The Dermal Papilla

The term dermal papilla should be used to designate only the connective tissue element which is enclosed by the bulb of the follicle during anagen, and which forms a compact ball of dermal cells underneath the "hair germ" during telogen. The dermal papilla is attached to the connective tissue sheath by a basal stalk. It is richly vascularized in large hair follicles, less so in smaller ones and not at all in the follicles of lanugo hairs

The papilla is pointed at its summit (Fig 3). In the follicles of pili multigemini the papilla is split into two or more parts, ranging from one papilla with two apices to completely separated papillae

In a growing follicle, the papilla is voluminous and the cells are far apart. The nuclei are large and ovoid and stain a pale color. The cytoplasm, which is vacuolated, stains lightly with basic dyes Between the cells is the hyaline ground substance and a loose argyrophilic framework In the resting follicle, the dermal papilla is a flattened, compact ball of cells with dense, round nuclei and barely visible cytoplasm Some pigment granules may be scattered between the cells It is generally believed that during catagen there is a reduction in the number of papilla cells, and that in early anagen the cells divide and increase in number (Wolbach, 1951) However, degeneration of papilla cells is rare, and cells remain intact even after X-irradiation (Montagna and Chase, 1956). The occasional mitotic figures found in the papillae of

human hair follicles usually prove to be in the nuclei of endothelial cells The number of papilla cells remains constant, and what changes do occur are mostly due to fluctuations in the number of endothelial cells, to the changes in the size of its cells, and to the changes in the intercellular substances. During the growth of hair the dermal papilla stains metachromatically, is periodic acid-Schiff-reactive, and contains some alkaline phosphatase. During periods of follicular quiescence these properties are lacking. The details of these changes will be described in subsequent chapters.

IV. Growth and Differentiation

Growth of hair is achieved by the proliferation of cells in the matrix and by their increase in volume as they move up into the upper bulb. The over-all architecture of the follicle may play some role in the dynamics of growth. The mass of cells in the bulb is funneled through a relatively narrow neck, maintaining the rate of growth of the hair with greater ease than if the matrix and upper bulb were of the same diameter as the hair. The cells may be under pressure as they pass through the neck of the bulb, and there must be a constraining mechanism to funnel them upward and to keep them from expanding laterally. Henle's layer of the inner sheath, the first rigid part of the funnel, becomes keratinized precociously just above the critical level The outer sheath, the vitreous membrane, and the connective tissue sheath give the funnel resiliency and firmness The cells outline patterns of shearing lines as they move up from the matrix, but these physical factors may be fortuitous The reconstructed models show that the back and the scalp contain true compound follicles, in which several hairs, each produced by a separate follicle, share a common orifice to the outside. Multiple beard hairs are occasionally produced by a single follicle, these hairs may range from single ones with a doubled medulla, to conjoined or completely separate hairs (Giovannini, 1907, 1908, 1909, 1910) When such pili multigemini occur, there is a corresponding duplication of the dermal papilla of the follicle (Pinkus, 1951) Such close correspondence between the subdivisions of the papilla and the number of hairs formed in a single follicle focuses attention on the dermal papilla as the inductive agent Each lobe of the papilla in such follicles induces hair formation from *a common matrix on its own accord, with each hair surrounded by its* own inner sheath, but with all the hairs sharing the same outer sheath This also points out the fact that the matrix gives rise only to the hair and the inner sheath, the outer sheath is self-propagating.

At the completion of its growth cycle, a follicle forms a club hair above the bulb, and the bulb largely degenerates At the onset of

catagen, mitotic activity in the matrix stops. The hair root becomes clavate with numerous keratinized fibers radiating from it. The club becomes surrounded by a capsule of partially keratinized cells, around which is a variably thick epithelial sac of indifferent epidermal cells. Below the epithelial sac, in place of the bulb is a strand of undifferentiated cells Most of the cells in the center of the strand degenerate, and the follicle becomes shorter. Scattered among the cells of the strand and the epithelial sac are a few remaining pigment cells. The matrix has degenerated and the dermal papilla is free, but remains in contact with the base of the epidermal strand (Fig. 8). At the beginning of catagen, the connective tissue sheath around the lower part of the follicle and the inner layer of the glassy membrane become very thick. When the strand is formed, the connective tissue sheath hangs loosely around it The retreat of the follicle upward seems to be brought about by the orderly progressive degeneration of cells both in the epithelial sac around the club hair and in the epidermal strand beneath it. The cord of cells and the lower layer of the epithelial sac comprise the hair germ

The cycles of growth of each follicle consist of the building up and tearing down of the structure After a period of rest, the follicle is built anew from raw materials, and each hair follicle goes through the identical processes The process of degeneration that takes place in catagen follows a profoundly orderly pattern. If such degeneration were brought about only by impaired nutrition, the cells would die all at once (Glucksmann, 1951) This is retrograde morphogenetic transformation and not degeneration in the usual sense.

The cycles of growth and rest, as well as the rate of growth of hair, vary in different follicles and in different regions of the body. In man, each follicle has its own cycles of growth, more or less independent of neighboring ones. With minor differences attributable to age, sex, and particular region, hair grows approximately 0 35 mm per day (Myers and Hamilton, 1951) The quiescent periods of individual follicles may be very short, as in those of the scalp, the beard, and the axilla, or very long, as in those of the general body surface

When the club hairs are plucked, new hairs begin to grow at once, clipping and shaving have no effect on growth The time required for human follicles to regrow hairs varies from 147 days for the hairs of the scalp to 61 days for those of the eyebrows (Myers and Hamilton, 1951) Substantial information about this, however, is lacking When a growing hair is plucked from the scalp, nearly all of the lower half of the follicle is pulled out with it The recovery period of such follicles is necessarily very long.

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CHAPTER 4

The Histochemistry of the Hair Follicle

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I. INTRODUCTION

The aim of histochemistry is to demonstrate certain compounds in cells and tissues and to arrive at conclusions about functional phenomena. Histochemistry has developed rapidly in the last few years. This becomes obvious when we consider the recent increase in the number of histochemical investigations dealing with the skin of animals and man Montagna did great service when he clearly summarized our knowledge of the histochemistry of normal skin in his monograph on "The Structure and Function of Skin" (1956). In this chapter we shall discuss briefly the results obtained with histochemical investigations of the hair follicle It will not be possible to discuss individual methods.

II. Histotopography of Inorganic Substances

When microincinerated, hair follicles are found to be rich in mineral ash. The nuclei of the hair cuticle contain abundant calcium, but the hair does not (Gans and Pakheiser, 1924). The hair follicle is exceptionally rich in potassium, and contains magnesium, calcium phosphate, and sulfate groups (Cornbleet, 1941; Herrmann, 1932). Unfortunately, except for iron, a precise localization and recognition of mineral ashes in tissues is very difficult and not reliable (Montagna, 1956).

Zinc is localized in the outer root sheath of hair follicles, but not in the inner root sheath and the cuticle. The outer root sheath in hair follicles of the guinea pig has large quantities of zinc (Braun-Falco and Rathjens, 1956a). In zinc insufficiencies in the rat and in the pig, the hair follicles atrophy (Follis *et al.*, 1941; Kernkamp and Ferrin, 1953) In psoriasis, the epidermis contains comparatively small amounts of zinc, but the hair follicles are normal (Braun-Falco and Rathjens, 1956b).

It would be of great interest to demonstrate copper in hair follicles, since it plays an important catalytic role in the formation of disulfide bonds (Rothman, 1954, 1955), but neither the technique of Mallory and Parker (1939) nor that of Okamoto and Utamura (1938) are sensitive enough to reveal it.

III. Histotopography of Carbohydrates

A Glycogen

The outer root sheath of active hair follicles is laden with glycogen (Fig 1), and the greatest concentration is found in the middle third (Sasakawa, 1921, Montagna, 1956) where the cells are so full of it that they appear to contain large vacuoles in ordinary histological preparations. The upper and lower thirds of the outer root sheath contain less glycogen The cells of the inner root sheath are practically free of it even before they become keratinized in the bulb, this is perhaps related *to the* precocious keratinization of these cells The cells of the cuticle of the cortex can be easily recognized above the bulb, glycogen decreases and [illegible] progresses The cells of the [illegible] the cortex, and, as in the cortex, it disappears as the cells become keratinized. The matrix cells in the bulb are always free of glycogen

The content of glycogen changes during the hair cycle (Shipman *et al.*, 1955). In the mouse, glycogen begins to increase in anagen III and reaches a maximum between anagens V and VI and disappears abruptly in catagen The cells of resting follicles in the mouse show no glycogen but those of man still contain some (Montagna *et al*, 1952)

The dermal papilla contains no glycogen. During catagen, or after x-irradiation, large amounts of it can be found in the papilla cells and in the ground substance (Montagna and Chase, 1956). The papilla is rich in periodic acid-Schiff (PAS)-positive material that is resistant to diastase (Fig 2); this substance, in contrast to the acid mucopolysaccharides and to alkaline phosphatase, does not disappear from the papilla of resting follicles.

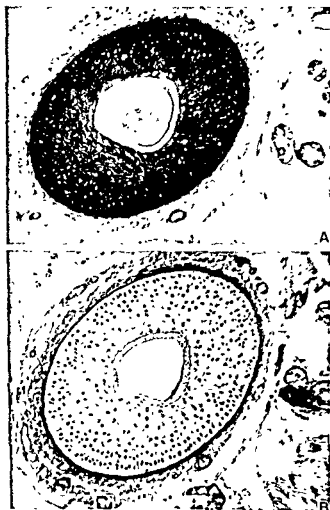


FIG 1 A Transverse section through a human hair follicle, showing much glycogen in the cells of the outer root sheath B Same follicle as in A with the glycogen removed by saliva digestion (Courtesy of Dr W Montagna)

Mitotically active cells or keratinizing cells are always free of glycogen (Montagna, 1956). The cells of the basal layer of normal skin epidermis contain no glycogen; those of the outer layers of squamous cell carcinomas are also free of glycogen, but the less mitotically active cells toward the center of tumors contain great quantities of it (Braun-Falco, 1954). Glycogen in the cells of the outer root sheath may indicate that these are practically inert during hair growth.

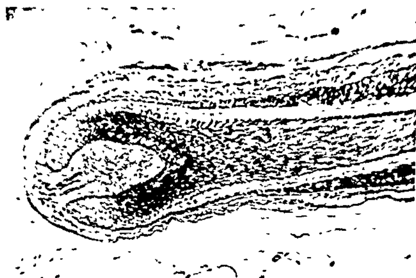


FIG 2 Strong PAS reaction in the connective tissue sheath and dermal papilla of a hair follicle from the scalp (Courtesy of Dr. W Montagna)

Glycogen in hair follicles in distributed inversely to the rate of keratinization, when keratinization begins in cells, glycogen disappears Montagna (1956) assumes that the glycogen in the outer root sheath is *the source of energy for protein synthesis during hair growth*, but this assumption is premature.

B Acid Mucopolysaccharides

Metachromatic staining, often masked by the intense basophilia of the cells of hair follicles, occurs mostly in the peripheral cell layers of the outer root sheath, which are also rich in glycogen The cells of the hair bulb, at the border of the dermal papilla, also show intense metachromasia The dermal papilla is strongly metachromatic in growing hair follicles but orthochromatic in quiescent ones The same material also is strongly positive with Alcian blue or Hale's reaction Based on enzymological tests, Montagna *et al* (1951b, 1952) suggest that this

metachromatic material is a sulfate-containing mucopolysaccharide, *probably chondroitin sulfate B* Sylvén (1950) believes that acid mucopolysaccharides play a role in supplying sulfur for keratinization and that they are labile sulfur-containing substances. He also believes that these substances are derived from the dermal papilla, which in growing hair follicles shows strong metachromasia (Montagna *et al*, 1951a; Sylvén, 1950). The connective tissue sheath of hair follicles stains metachromatically. The glassy membrane is strongly PAS-positive and behaves histochemically like the PAS-reactive subepidermal basement membrane (Braun-Falco, 1955).

IV. The Histotopography of Lipids

Few have investigated histochemically the lipids in hair follicles. The cells of hair follicles contain only traces of sudanophilic lipids (Nicolau, 1911). The outer root sheath is said to contain phosphatides and cholesterol, and the inner root sheath cholesterol, glycerol esters, soaps of fatty acids, and glycerol Montagna *et al* (1951a) found sudanophilic granules with an eccentric, sudanophobe vacuole in all of the nonkeratinized cells of active hair follicles located perinuclearly and bunched in the upper pole of the nucleus. Kreibich (1922) also observed *perinuclear fat granules often connected in a chainlike fashion* in the region of the Golgi bodies in epidermal cells. The nodules of Bizzozero in the cells of the outer root sheath are weakly sudanophilic (Dupré, 1952) The cells of the pilary canal, beginning at the level of the sebaceous glands, have sudanophil granules that correspond to the Golgi apparatus (Montagna, 1950) or lipochondria (Baker, 1950).

Discrete lipid granules are found in the cells of the presumptive inner root sheath in the upper portion of the bulb, these disappear when trichohyalin forms in these cells (Montagna, 1950, 1956). The fragmented inner root sheath becomes strongly positive because it is impregnated with sebum at the orifice of the sebaceous glands The inner root sheath also is sudanophilic below the level of the orifice of the pilosebaceous canals The keratinized cortex and the medulla of hairs are relatively free from lipids The papilla cells contain only traces of lipids With Nile blue sulfate, the entire hyalinized inner root sheath stains blue, the sebum, which contains neutral fat, stains a rose pink The blue reaction in the inner root sheath may indicate the presence of acid lipids. With Baker's acid hematein method, the inner root sheath and the matrix cells are shown to contain much phospholipid The amount of these substances in the hair root decreases with the commencing of keratinization, this is slower in the cells of the medulla than in those of the cortex The distribution of these lipids demonstrates that the lipids

are an intrinsic part of the cells, and that phospholipids seem to play an important role in the formation of hair keratin and inner root sheath trichohyalin.

In resting hair follicles, nearly all of the cells of the epithelial sac contain small sudanophilic granules, but the cells of the hair germ and those of the papilla possess none (Montagna, 1956).

Great amounts of plasmal can be demonstrated in human hair follicles with the method of Feulgen and Voit (1924). An intense pink stain, mainly in the cells of the outer root sheath, is strongest in the peripheral cells. This is in agreement with findings in the epidermis, where the lower cell layers of
but the cells in the upper layer
also can be shown in the cells of the hair matrix. The hyalinized inner root sheath and the keratinized medulla are free of plasmal

V. Amino Acids and Proteins

The available histochemical methods for the demonstration of proteins are relatively crude. The demonstration of simple proteins is possible only in a limited way. For the study of amino acids, the Millon reaction and the tetrazonium method of Danielli (1947) are used for tyrosine, the technique of Sakaguchi (1950) for arginine (Steigleder, 1957), that of Wieland and Bauer (1955) for tryptophan, none of these methods give convincing results The inner root sheath is rich in arginine The medulla of the hair contains greater quantities of tyrosine than any other part of the follicle (Giroud *et al.*, 1934; Stoves, 1953) Hair keratin contains tryptophan The alloxan and ninhydrin-Schiff reaction of Burstone (1955) shows only the free alpha amino acids, and not those bound in dipeptide chains. In the active human hair follicle, besides a weak cytoplasmic reaction in all of the cells, there is an intense positive reaction in the keratogenous zone, which corresponds to the region rich in sulfhydryl groups The keratogenous zone has great concentration of free amino acids, probably as a result of protein synthesis connected with an increase of short-chained proteins The fully formed hair is free of histochemically demonstrable amino acids, but cells of the outer root sheath, directly bordering the hyalinized inner root sheath, abound in them.

VI Substances Containing Sulfhydryl and Disulfide Groups

Protein-bound sulfhydryl groups are important in the production of hair keratin (Giroud and Bulliard, 1933, 1934, Giroud and Leblond, 1951) They are also necessary for the activity of many enzymes, and function as chemical bonds between proteins and their prosthetic groups

Several authors have investigated the distribution of the thiol groups

in the hair follicle. Buffa (1904) found them in the hair bulb and in the root sheaths. Kaye (1924) found a strong positive —SH reaction in the hair follicle and in the hair root of the ox. Walker (1925), and Giroud and Bulliard (1933) called attention to the inverse relationship between increasing keratinization and the content of —SH groups. Barrnett (1953) found a uniform distribution of —SH groups in the outer root sheath, in the cuticles of the hair, and the inner sheath, and less of it in the inner root sheath in the rat. The cells of the bulb also have a moderate amount of —SH groups, and the dermal papilla has very little of it. In the keratinizing cortex of the hair, the reaction is progressively more strongly positive, up to about one-third of the hair shaft, comprising the keratogenous zone (Fig. 3). Distal to the keratogenous zone,

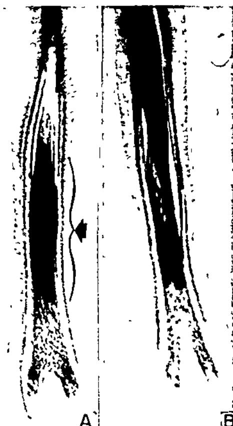


FIG. 3 A Longitudinal section of a hair follicle from the scalp, treated with the Barrnett method for —SH group Only the keratogenous zone, within the bracket, is strongly reactive B. Section of the same follicle as in A, showing —S—S— groups in the hair shaft

no —SH groups are found. A positive reaction in the medulla extends farther up in the hair than the keratogenous zone. In the skin of the mouse and guinea pig, there is an increase of —SH groups in the cells of the outer root sheath during early anagen (Eisen *et al.*, 1953). The cortex of the fully keratinized hair is negative, but the cuticle remains positive up to the level of the pilary canal. The inner root sheath and the medulla also contain —SH groups. In catagen the brush of the keratinizing hair club is rich in —SH groups, while the outer and inner root sheaths contain more —SH groups than during anagen.

The distribution of —SH groups in human follicles is similar to that



FIG 4 A —SH reaction in the cells of the inner root sheath, the trichohyalin granules are negative B —SH reaction in the keratogenous zone The upper part is strongly reactive, reactive fibrils can be seen in the lower part of the keratogenous zone (Courtesy of Dr. W Montagna)

of the mouse and guinea pig (Figs 3 and 4) (Montagna *et al.*, 1954, Odland, 1953, Foraker and Wingo, 1955). A strong positive reaction is found in the outer root sheath, in the inner root sheath, Henle's layer is more intensely positive than Huxley's layer. Trichohyalin is always free of —SH groups, the intercellular bridges and the nodules of Bizzozero are strongly positive, indicating that the tonofibrils are the anlagen of keratin The reaction is particularly strong in the keratogenous zone of the hair root In the resting follicle, the epithelial sac, containing the hair club, is weakly positive, while the club itself shows streaks of reaction. There is a finely fibrous and intensely positive zone between the cells of the epithelial sac and the keratinized hair club The hair

germ, which extends from the epithelial sac to the resting papilla, is only weakly positive (Montagna, 1956).

The distribution of protein-bound disulfide groups is similar to that of the thiol groups, except that the keratinized hair cortex is the most strongly positive element (Montagna, 1956); the club in a resting follicle is weakly reactive.

VII. Nucleic Acids

Abundant ribonucleic acid (RNA) is characteristic of those cells that carry out appreciable protein synthesis Deoxyribonucleic acid (DNA) is found only in the nucleus Like that of RNA, the content of DNA

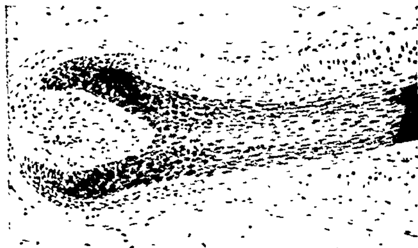


Fig 5 Distribution of DNA in a growing follicle from the scalp (Courtesy of Dr W. Montagna)

increases in cells during division An increase in DNA content appears to be indirectly related to an increase in protein synthesis The nucleus is supposed to control the number of microsomes which, probably by autoduplication, represent the agents responsible for protein synthesis (Brachet, 1952)

Deoxyribonucleic acid is demonstrated with the Feulgen reaction. At the beginning of each hair cycle, most of the cells of active hair follicles have nuclei that are uniformly strongly Feulgen reactive, but the nuclei of the mitotically active cells react the strongest (Fig. 5) With the beginning of keratinization the differentiating cells progressively lose their stainability (Montagna, 1956, Hardy, 1952) During the early active stages, the nuclei of the papilla cells are strongly positive Taking into consideration that the DNA content is constant in a diploid

cell (Mirsky and Ris, 1949, Schrader and Leuchtenberger, 1950), the changes *in the intensity of Feulgen reaction is likely to be connected* with differences in the nuclear volume.

In an active follicle the nuclei of the undifferentiated cells of the matrix possess the greatest quantity of RNA. The differentiating cells of the hair cortex retain strong concentrations of RNA up to the lower part of the keratogenous zone, where active protein synthesis ceases, and RNA disappears abruptly. Birefringence is increased here, indicating an increasing orientation of submicroscopic aggregates of molecules, and also an increase in protein-bound thiol groups. The other parts of the follicle have smaller amounts of RNA In the slowly keratinizing cells, some RNA can be seen up to the middle third of the follicle. In the inner root sheath, RNA decreases and disappears with increasing hyalinization In completely hyalinized inner root sheath, however, there still is RNA demonstrable.

The distribution of RNA in the hair follicle shows that this substance is closely linked with the rates of protein synthesis. Ribonucleic acid disappears from the cells of the bulb at the same pace that the formation of protein fibrils increases in them, and is related to the keratinizing activity of the hair follicle

VIII. The Histotopography of Enzymes

The aim of enzyme histochemistry is to obtain a topographic localization of enzymes in tissues Unfortunately, histochemical methods employ unphysiologically high concentrations of substrates In living tissues, the amounts of enzymes present surpasses the necessary minimum for normal metabolism, and is up to 600 times higher than is needed (Siebert, 1956), and an inhibition of about 99% would not necessarily affect the rate of metabolism Furthermore, the actual metabolism may be normal even in tissues where histochemically only a weak enzymatic activity can be demonstrated Consequently, the results of enzyme histochemistry should be interpreted with the greatest caution (Siebert, 1956).

A Phosphorylase

Most of the energy for metabolic synthesis and secretion is derived from the physiological breakdown of carbohydrates, fats, and proteins The intermediary carbohydrate metabolism comprises all enzymatic reactions that take part in the breakdown of glycogen through glucose *into carbon dioxide and water The two known phases are anaerobic* glycolysis and the biological oxidation in the Krebs cycle. Fat and protein are also oxidized in the Krebs cycle after they have been split

In contrast to biolgoical oxidation, anaerobic glycolysis provides only a small amount of the total phosphorus-bound energy (Lardy, 1949).

Since a series of enzymes of the anaerobic glycolysis have been demonstrated in skin (Rothman, 1954), there must be a glycolysis chain, although it is not yet posssible to demonstrate histochemically each step in the reaction In the last step for glycogen synthesis, the reaction glucose-1-phosphate, glycogen is reversibly catalyzed with the enzyme phosphorylase. If the skin is incubated in glucose-1-phosphate with the addition of such activators as insulin, muscle adenylic acid, and glycogen, the tissue-specific phosphorylase forms a polysaccharide which stains blue with Lugol's iodine solution (Takeuchi and Kuriaki's method,



FIG 6 A Phosphorylase activity in hair follicles and epidermis B Phosphorylase activity in the outer root sheath (arrows) of human hair follicles (Courtesy of Dr R A Ellis)

1955) *Such a polysaccharide is probably a chain polymer of amylose* type (Braun-Falco, 1956a). The unkeratinized cells of the hair follicle, particularly in the bulb, possess strong phosphorylase activity (Fig. 6a). The cells of the outer root sheath (Fig. 6b), particularly in the middle and upper thirds of the follicle, and those of Henle's layer, are strongly positive (Braun-Falco, 1956a). *All living epidermal cells, then, are* capable of synthesizing glycogen from glucose-1-phosphate. It can be *assumed that an increase of glycogen synthesis in cells takes place when* particular energy-demanding functions are performed

B Aldolase

The enzyme, aldolase, catalyzes the reaction that splits fructose-1,6-diphosphate into glycerin-aldehyde phosphate and dioxyacetophosphate. Although biochemical methods have demonstrated aldolase activity in the epidermis (Wust, 1956), Spier and Martin (1956) have not succeeded in demonstrating this enzyme in hair follicles with the technique of Allen and Bourne (1943). In pilot experiments, we have demonstrated *this enzyme with an azo-coupling technique in the hair follicle, but these* investigations are not yet completed

C. Succinic Dehydrogenase

Succinic dehydrogenase, the most investigated enzyme of the Krebs cycle, is *topographically related to the regions of cell growth and energy-*demanding cell functions. Colored intracellular formazan crystals are formed from various tetrazolium compounds at the sites of succinic dehydrogenase activity.

Enzyme activity has been observed in the hair follicle of the sheep, *mouse, rat, guinea pig, rabbit, dog, and man (Formisano and Montagna,* 1954, Braun-Falco and Rathjens, 1954a, Padykula, 1952, Montagna and Formisano, *1955a, Serri, 1955)*, and the distribution is similar in all of these species During the resting phase of the hair growth cycle the follicles show little enzyme activity (Argyris, 1956, Padykula, 1952, Formisano and Montagna, 1954) In active follicles the highest enzyme activity is in the matrix of the hair bulb The cells in the upper bulb *and in the dermal papilla also show enzyme activity*, activity decreases and ceases in the keratogenous zone (Fig. 7a) The glycogen-rich outer *root sheath shows enzyme concentration, particularly in the* upper part of the hair follicle. The cells of the inner root sheath are weakly active in the bulb, while those in the hyalinized portion are negative

D. *Cytochrome Oxidase*

Rothman (1954) has described in detail the biochemical importance in the skin of cytochrome oxidase, the yellow respiratory enzyme of Warburg Using the nadi-indophenol blue technique (Graff, 1949), the histochemical distribution of this enzyme corresponds to that of succinic dehydrogenase. The cells of the matrix are the most strongly positive The reaction is positive also in the outer root sheath and in the hair bulb, while the more distal portions of the follicle are negative (Rogers, 1953).

E. *Monoamino Oxidase*

Hair follicles are only weakly positive for monoamino oxidase (Shelley *et al.*, 1955). The special task of this enzyme is not engaged in the general metabolism

a. Oxidases in melanin formation The histotopography of the oxidases in melanin formation are discussed elsewhere in this book by Fitzpatrick *et al.* (Chapter 13).

F *Esterases*

In this group of enzymes belong the esterases that act on inorganic esters, i e., phosphomonoesterases, alkaline and acid phosphatases, and the large groups of esterases that act on organic esters, *i e.*, lipases, cholinesterases, and others.

1. *Phosphatases*

Phosphatases play an important role in the intermediary metabolism of carbohydrates and in the synthesis and breakdown of nucleic acids Transphosphorylation may occur in the presence of adenylic acid systems under the action of alkaline phosphatases (Lang, 1952). It is remarkable that alkaline phosphatase is not present in all metabolically active organs (Spier and van Caneghem, 1957). This, however, could be explained by the fact that the liberation of inorganic phosphate is a subordinated phenomenon *in vivo*

For the demonstration of phosphatases, either the azo-dye methods, or the method of Gomori (1945) are used, the techniques, however, have different sensitivities (Moretti and Mescon, 1956a, b)

a Acid phosphatase. The distribution of this enzyme in hair follicles is somewhat complicated (Moretti and Mescon, 1956a, Spier and Martin, 1956) Similar distribution of acid phosphatase can be demonstrated with various methods using different substrates A positive reaction is obtained in the cells of the lower bulb, the proximal portion of the medulla, and the inner root sheath, the intensity of the reaction increases distally toward the hair (Figs 7b, c, d) The outer root sheath

2 Esterases Acting on Esters of Organic Acids

Two groups of enzymes, aliesterases, and cholinesterases belong to this category (Gomori, 1939, 1945). The aliesterases are the simple, or nonspecific esterases, which hydrolyze the esters of short-chained fatty acids with simple alcohols, and the lipases, which split the esters of long-chained fatty acids with glycerin Lipases are activated with sodium taurocholate, whereas the simple esterases are inhibited by it (Nachlas and Seligman, 1949a).

Nonspecific esterases can be demonstrated with the azo-dye reaction, using α-naphthylacetate or α-naphthylbutyrate as substrates (Nachlas and Seligman, 1949b, Gomori, 1945, Pearse, 1954a, b), and also with the indoxyl method (Barrnett and Seligman, 1951). The Tween method demonstrates the lipases (Gomori, 1949).

The hair follicles of rabbits and guinea pigs show only little esterase activity in comparison with those of the mouse, rat, cat, and pig (Montagna and Formisano, 1955b). There is surprisingly little nonspecific esterase activity in the keratogenous zone of hair follicles Enzyme activity is strongest in the lower half of the follicle and is confined mainly to the outer root sheath. There is greater enzyme activity in the growing than in the quiescent follicle.

Human hair follicles also show appreciable esterase activity (Braun-Falco, 1956c, Findlay, 1955a, Montagna, 1956, Steigleder and Loffler, 1956). The outer root sheath is strongly positive, while the activity in the inner root sheath, contrary to the assertion of Findlay (1955a) is weak. The dermal papilla probably does not react at all. The degenerating cells of the bulb are rich in esterases during catagen (Montagna, 1956), a situation corresponding also to that of the mouse (Argyris, 1956) In the resting phase, the outer root sheath shows only weak activity, except for a few strongly positive cells in the epithelial capsule. When eserine ($2 \times 10^{-6} M$) is added to the indoxyl acetate method, the esterases are not inhibited (Braun-Falco, 1956b) This means that cholinesterase is not a part of the reactive enzyme in the hair follicles Since sodium taurocholate and eserine ($10^{-4} M$) partially inhibit the indoxyl reaction in the follicle (Braun-Falco, 1956b), one should assume that lipases as well as nonspecific esterases are present in hair follicles Steigleder (1956b) did not succeed in demonstrating lipase in follicles with the Tween method of Gomori

Considering the distribution of esterases in normal skin, the rich zone under the stratum corneum is compelling evidence of the relation of esterases to keratinization in the epidermis. It is surprising, therefore, not to find such a parallel in hair follicles.

3 *Cholinesterase*

The histotopography of cholinesterase is discussed by Montagna and Ellis on page 224 of this book.

G. *β-Glucuronidase*

Beside hydrolyzing conjugated glucuronide, β-glucuronidase is probably related to cell proliferation. Its role in coupling estrogen with glucuronic acid is also important (Fishman *et al*, 1954) β-Glucuronidase catalyzes the breakdown of disaccharides to monosaccharides. The disaccharides arise from hyaluronic acid as split products through the action of hyaluronidase (Meyer *et al*, 1951).

In human hair follicles, β-glucuronidase is concentrated in the hair bulb, enzymatic activity decreases in the keratogenous zone. There is also a strong reaction in the inner and outer root sheaths. The keratinized structures are all negative (Braun-Falco, 1956g).

We have only hypothetical knowledge of the biological activity of β-glucuronidase. The increased activity in the upper portion of the epidermis and in the inner root sheath indicate that this enzyme is important in keratinization.

H. *Carbonic Anhydrase*

Carbonic anhydrase catalyzes the reaction. $CO_2 + H_2O \rightarrow H_2CO_3$. It is particularly important in gas exchange in the erythrocytes, where the hydration of incorporated molecular CO_2 is greatly speeded up. Carbonic anhydrase activity is responsible for the production of hydrochloric acid in the cells of the gastric glands, in the kidney carbonic anhydrase strongly influences the electrolyte composition of the urine. In skin, the greatest activity is found in the ducts of sweat glands (Braun-Falco and Rathjens, 1955b). Hair follicles have a weak enzymatic activity, and its significance in hair growth is not known.

I *The Aminopeptidases*

The breakdown of proteins is parallel with synthesis In skin, keratin is continuously synthesized, and intense peptidase activity would be expected to be found in the keratogenous zone of hair follicles, where the important synthesizing processes take place The histochemical demonstration of proteinase and peptidase activity is relatively difficult, but good results can be obtained with the freeze-drying technique and with frozen sections (Braun-Falco, 1956c)

Surprisingly, aminopeptidase is not particularly heavily concentrated in the keratogenous zone (Braun-Falco, 1956c), a stronger activity is

found in the marginal zone of the outer root sheath and in the matrix of the bulb. In the upper two-thirds of the follicle, aminopeptidase activity is limited to the outer root sheath (Fig. 8a). Because of the solubility of azo-dyes in lipoids, there is a diffuse, pink coloration in the hyalinized inner root sheath; this is an artifact. The dermal papilla shows a little enzymatic activity.

The histotopography of aminopeptidase indicates that this enzyme is not particularly important in the process of keratinization. This is understandable, if we consider that the reversal of the hydrolytic effect of peptidases is very unlikely on thermodynamical grounds, and that



FIG 8 A Aminopeptidase activity in the epidermis, and in the outer root sheath of hair follicles (arrows) B Hair keratin stained with the peracetic acid-aldehyde-fuchsin reaction showing a positive reaction.

the biosynthesis of peptides from amino acids takes place with other enzymes utilizing phosphate energy (Hoffmann-Ostenhof, 1954).

This enzyme is present in normal epidermis and hair follicle in an inhibited state and is liberated only when damage occurs. For instance, there is an intense epidermal aminopeptidase activity in the beginning of vesicle formation in dermatitis and in pemphigus (Braun-Falco, 1956c, 1957) Aminopeptidase may play a role in the exchange of materials between the epithelium and the connective tissue

IX. HISTOCHEMISTRY OF KERATIN OF THE HAIR CORTEX

Keratin is characterized by high concentrations of the basic amino acids arginine, lysine, and histidine, and the sulfur-containing amino acid cystine The identification of formed keratin by its resistance to trypsin and pepsin is of some value (Pearse, 1954b) The method of

Burstone (1955) for demonstrating free amino acids, particularly the alloxan and ninhydrin-Schiff technique, is not suitable for the demonstration of the fully keratinized hair cortex; however, the reaction is positive only in the keratogenous zone, where the content of —SH groups is very high, and where the incorporation of radioactive cystine is the strongest (Bern, 1954, Bern *et al*, 1955) This seems to indicate that the fully keratinized hair cortex contains only a small amount of free amino acid

The technique of Sakaguchi (1950) for the demonstration of arginine, modified by Warren and McManus (1951), is very specific but not of great value in differentiating between strong and weak positivity, this technique acts in a strong alkaline solution and affects the tissues adversely (Pearse, 1954b).

The demonstration of tyrosine with the Millon reagent does not give convincing results Tyrosine is said to be more abundant in the medulla than in the rest of the hair (Stoves, 1953). With the tetrazolium-coupling method (Danielli, 1947), tyrosine is found mostly in the hair keratin, because only small amounts of histidine and tryptophan are present in the hair (Block, 1939).

The methods for the demonstration of —S—S— containing compounds in hair are very useful, the method of Barrnett-Seligman (1954) reaction gives good results

Hair keratin is normally not basophilic when stained with basic dyes buffered to pH 4 or below. Strong basophilia develops in the hair shaft after it is treated with periodic acid, potassium permanganate, and chromic acid, this has been explained to be due to sulfonic acids (Dempsey *et al*, 1950) After oxidation with peracetic acid, or treatment with bromium or neutral potassium permanganate, a strongly acidic compound develops in the hair keratin, which takes basic dyes even at pH 1 2 (Lillie *et al*, 1954, Lillie and Bangle, 1954) This is more strongly resistant to methylation as are the basophil substances in mast cells and in cartilage It is likely that the resulting metachromatic basophilia is connected with the oxidative split of disulfide groups, with the formation of cysteic acid. The rest of the cysteic acid is not free in the oxidized keratin, but is bound in peptide chains, and therefore does not go easily into solution (Lillie *et al*, 1954).

Oxidation of keratin with peracetic acid does not cause a splitting of peptide chains and the liberation of carboxyl groups (Alexander *et al*, 1951) After peptides are split with such strong oxidizing agents as potassium permanganate, there is loss of —NH_2 groups and the liberation of numerous carboxyl groups, which could give rise to metachromatic staining.

Hair keratin is stained red with the Schiff reagent after previous oxidation with performic acid or peracetic acid (Pearse, 1951), or after long oxidation with acid $KMnO_4$ (Braun-Falco and Rathjens, 1954a, b, 1955a, b). The disulfide bonds in hair are said to undergo oxidative splitting with peracetic oxidation, and the newly formed alanine-1-sulfinic acids, representing an intermediary product in the oxidation of cystine to cystinic acid, are reactive to the Schiff reagent Neither acetylation nor previous bromination influence the results of the reaction with performic acid or with the Schiff reagent (Braun-Falco and Rathjens, 1954a, b) This indicates that neither α-glycol nor α-aminoalcohol and other ethylenic groups could be responsible for a positive reaction in the hair keratin, and aldehydes do not play an important role in the positive reaction (Findlay, 1955b). An unsaturated, insoluble complex may be responsible for a positive peracetic-Schiff reaction in the hair keratin (Lillie and Bangle, 1954). A positive performic acid-Schiff reaction in the hair keratin is bound to the presence of disulfide linkages and not to the oxidation products of —SH groups in tissues (Braun-Falco and Rathjens, 1954a, b) A performic acid-Schiff reaction is negative in the stratum corneum, when —S—S— groups are first transformed into —SH groups with thioglycolic acid. If, before applying the performic acid-Schiff reaction, the —SH groups in the epidermis and hair follicle are oxidized into —S—S— groups with potassium iodide, they are also positive The oxidation products positive to the Schiff reaction could be disulfoxide and disulfone.

The aldehyde-fuchsin reaction of Gomori (1950) is suitable for the demonstration of hair keratin that has been previously treated with peracetic acid (Fig 8b) (Scott, 1953) or acid potassium permanganate (Braun-Falco and Rathjens, 1954a, b, 1955a, b) Besides keratin, elastic fibers and mast cells are also stainable with this method, indicating that mainly sulfur-containing tissues react After previous sulfonation, collagen is also positive with aldehyde fuchsin (cf Braun-Falco, 1956d, e) Pretreatment with acetylation, bromination, potassium iodide, and thioglycolic acid does not influence a positive performic acid or potassium permanganate-acid fuchsin reaction in the hair keratin (Braun-Falco and Rathjens, 1954b). The performic or peracetic acid and $KMnO_4$-Schiff technique from a histochemical point of view, therefore, cannot be compared with the performic or peracetic acid or $KMnO_4$-aldehyde-fuchsin method.

Keratin of epidermis can be demonstrated with the gram stain (Ernst, 1896), and the positive gram reaction may depend on —SH groups (Fischer, 1953). The keratin of hair and nails is negative. Gram-negative wool keratin becomes positive when treated with KOH and

with reducing compounds The gram-positivity of epidermal keratins can be abolished with a previous blocking by monoiodineacetate (Steigleder, 1956a). The blocking of —SH groups and the conversion of —S—S— groups into —SH groups results in a corresponding gram-staining.

In summary, although a series of techniques can be used to identify keratin, and particularly hair keratin, the majority of these methods are not absolutely specific.

X THE HISTOCHEMISTRY OF TRICHOHYALIN

All three layers of the inner root sheath contain hyaline granules, first described by Vorner (1903), and called trichohyalin. These take up basic dyes more quickly than acid stains and are isotropic Trichohyalin is more fuchsinophil than keratohyalin. When first formed, trichohyalin granules are relatively small, but later they unite into larger, round or rodlike, homogeneous structures. The cells of the inner root sheath finally become hyalinized and their nuclei shrink and disappear. During this process, the cells do not decrease in volume, probably because they are already saturated with trichohyalin (Montagna, 1956). Ribonucleic acid decreases in the cells of the inner root sheath as trichohyalin increases. These granules can be demonstrated well with the pyridine extraction method of Baker Lipids disappear in the cells when trichohyalin granules appear Trichohyalin has been regarded by some as an intermediary product of keratinization (Branca, 1911, Marston, 1946) It does not contain DNA or RNA, glycogen or other polysaccharides (Hardy, 1952), sulfhydryl or disulfide groups (Montagna *et al*, 1954), and it seems to have no enzyme activity Since the granules can be digested with trypsin, they may partly consist of proteins. Trichohyalin is deeply stained after incubation in dopa, pyrogallol, or adrenalin (Lillie, 1956). They are also stained with the polyphenolic dye gallocyanin after being exposed to it for 2 or 3 days, and with hematoxylin. This reaction seems to require oxygen and is inhibited by a stoichiometrical concentration of sodium sulfate or cysteine, which seem to compete for oxygen The polyphenol activity of trichohyalin is inhibited by formalin fixation but resists warm methanol-chloroform fixation and paraffin embedding

XI HISTOCHEMICAL ASPECTS OF THE KERATINIZATION OF THE HAIR

Keratinization of the hair cortex is a gradual process that is completed in the upper portions of the hair root. The formation of the fully keratinized hair is a process that takes part in the entire length of the hair root (Mercer, 1949) The most important transformation of the

polypeptide chains by the change of —SH to —S—S— bonds can be demonstrated histochemically. The high —SH group content in the fibrillar zone of the keratogenous zone may indicate that the folded polypeptide chains straighten out, and hidden —SH groups appear and disappear again as the formation of —S—S— groups increases (Rothman, 1954). During keratinogenesis there is also a new distribution of amino acids; hair keratin is richer in sulfur than the proteins in the still viable cells Sylvén (1950) regards the metachromatic material in the bulb cells of the growing follicle as the sulfur source for keratin synthesis, but this is not easy to accept since large amounts of this material are present in the outer root sheath, and there are practically none in the prekeratotic zone The localization of aminopeptidase also indicates a particular state of metabolism in the same zone. There is a hydrolytic split of amino acids simultaneously with keratinization, which is combined with a resynthesis of keratin (Rothman, 1954, 1955). The small concentration of aliphatic monoamino acids in keratin indicates that these are split from protein molecules, as there also occurs an apparent increase of —SH-containing radicals (Sammartino, 1922) Many free amino acids are recovered in cold aqueous extracts of hairs, but there are no sulfur-containing ones (Rothman, 1954) There is active protein transformation in the keratogenous zone of the hair follicle. The specific uptake of radio cystine in the keratogenous zone is also important in this connection (Bern, 1954, Bern *et al*, 1955). The pathway of radiocystine from the circulating blood into the keratogenous zone is not yet known Beside the absorption through the hair bulb, one should also consider the possibility of the transport through the capillaries around the follicle, the mucopolysaccharides in the bulb could also be a means of transport The considerable uptake of S^{35} in the follicle is also of importance (Bostrom *et al*, 1953). Consequently, it also becomes obvious that cells in the keratogenous zone form keratin *de novo*. Their nuclei, although moribund, still contain DNA and RNA. Thus, these cells may still play an important role in protein synthesis

In keratinization, copper seemingly catalyzes, as a coenzyme, the oxidative transformation of —SH into —S—S— bonds (Rothman, 1954).

Basophilic staining strongly decreases with increasing keratinization (Odland, 1953), whereas basic dyes are taken up in the fibrillation zone at pH 4 and higher, the fully keratinized cortex does not stain below pH 7 The decrease in basophilia is related to the disappearance of acid groups, as they build salt bridges between monobasic and dicarboxylic amino acids such as glutaminic acid and lysine These bridges break when the keratin swells in weak acids or alkalies (Rothman, 1954, 1955).

The uptake of acid dyes is practically the same in the various zones

of keratinization The fibrillar structures in the keratogenous zone, however, take up the dyes at pH 3 to 7. The earliest microscopically demonstrable fibers, then, are already similar to the hard cortex in their electrochemical nature, and the tonofibrils probably can be regarded as the precursors of keratinized structures

All physiological breakdown of nuclei is induced by an enzyme depolymerizing DNA, and there is increased DNA-depolymerase content in the keratogenous zone (Spier and van Caneghem, 1957), at the same time that the Feulgen reaction becomes negative. There is an intense concentration of acid phosphatases in the prekeratotic zone, where the physiological breakdown of the nuclei takes place. A small concentration of 5-nucleotidase is present in the keratogenous zone (Spier and Martin, 1956). In the inner root sheath, where less keratinization takes place, the loss of nuclei proceeds more slowly, and RNA can be demonstrated in the hyalinized portions of the root sheath. Histochemically we do not know the fate of the free breakdown products of the nucleoproteins, because purine cannot be demonstrated Aqueous extracts of hair contain pentoses, uric acid, and other purines in great quantity; these are certainly liberated from the nuclei as by-products during keratinization (Bolliger, 1951) The liberated purine transforms completely into uric acid. The high concentration of free amino acids in the hair may indicate that the histone of RNA also breaks down

The findings discussed here show that it is possible to throw some light on the complicated process of hair growth with histochemical methods. We are at the beginning of these investigations, and there are still great difficulties in methods These methods provide a valuable complement to histological-morphological findings. Only a meticulous investigation and an intimate contact between dermatological biochemistry and physiology, and histochemistry and biophysics can bring real progress to our knowledge.

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CHAPTER 5

The Electron Microscopy of Keratinized Tissues[1]

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I. INTRODUCTION

Electron microscopy has become an indispensable adjunct to cytology. When its findings are combined with those of classic histology and histochemistry, a complete picture of the structural elements of tissues and cells becomes possible, which must surely carry us a great way toward understanding their function Few organs lend themselves better to this integrated approach than skin and hair They are simple in structure and function relative to, let us say, liver or brain, and, mainly for economic reasons, have come in for a fair share of biological and physicochemical study yielding a vast amount of data to be correlated with fine structure

The first step of the electron microscopist is to identify already familiar structures by examining his materials in a manner immediately comparable with light microscopy Now that the method of preparing thin sections of biological material for use in the new instrument has been perfected, this step offers no particular difficulty Low magnification electron micrographs of sections may be directly compared with the light micrographs of the same sections and their common features identified When components have already been analyzed histo-

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chemically, this information may be directly read into the electron micrograph.

To obtain sections for most forms of microscopy, the material must be fixed and embedded. There are by now well-established standard techniques in electron microscopy (Palade, 1952, Rhodin, 1954, Ross, 1956) but, in our hands and in those of others, these procedures often gave poor results with keratinized tissues Fixation itself may not be satisfactory, apparently because the dermis and stratum corneum are not easily penetrated by the fixative Fixation of follicles is good, however, when these are plucked out of the skin and immediately immersed in the standard buffered osmium fixative (Palade, 1952). Keratinized skin remains difficult, but fair results have been obtained by placing a drop of fixative on the skin and immediately making a number of vertical cuts before the fragment is sliced off. Embedding leads to other defects. When methacrylates are used as the embedding substance, as is almost universal, the specimen may swell and actually fragment during polymerization. This type of damage ("explosion") is due to uneven polymerization, a local catalytic effect of the tissue, and causes low molecular weight monomers to enter and swell the polymer-impregnated tissue We have overcome this trouble by abandoning methacrylates in favor of an epoxide resin (Glauert *et al*, 1956)

Certain types of problems are more suitable for electron microscopy than others. Some are best solved by low magnifications and a view of many cells, others require the utmost resolution available. Among the latter are the fine scale cytological aspects of growth, differentiation, and synthesis The family of epidermal cells poses several such problems and the histology of the tissues is favorable for their solution. There are numerous specializations with a corresponding variety in differentiation and, in each instance, the life history of the cells, from division to disintegration, is recorded in a linear sequence which may be read off in a single section

The synthetic repertoire of the epidermis has become limited during development, but is sufficiently varied in that it includes several kinds of keratin, keratohyalin (trichohyalin), mucins, lipids, and a number of other materials produced in small amounts. Rudall has recently summarized some of the problems met with here (1956). The type of material which a particular cell produces in quantity depends upon the situation in which it finds itself, and the control seems to be effected largely by hormonelike compounds in the food supply Electron microscopy might be expected to enlarge our knowledge of the early fine structural changes accompanying differentiation and to reveal in greater detail the steps by which the syntheses are effected

Epidermal cells retain the products of their synthetic activities within their confines and seem to "choke themselves to death." Montagna (1956) has in fact likened the whole cutaneous system to a huge holocrine gland maintained by constant proliferation No stabilized permanent fabric of fixed secreting cells is produced, as in apocrine and epicrine glands, and this is reflected, as we shall see, in certain aspects of their intracellular fine structure.

There are some "keratins" which seem exceptional in being extracellular In some birds the gizzard is lined with a horny, secreted cuticle, said to be keratin. Also, bird and reptile eggs have membranes with a fibrous, keratinous component secreted by certain oviduct cells While these cells have not yet been examined, egg shell membrane keratin has proved to be composed of fibers quite different in appearance from intracellular keratin.

The discussion that follows will be of a comparative character, being based mainly on a study of the germinal tissues of skin, hair, and feathers. There is not a large literature dealing with the electron microscopy of these tissues which needs reviewing here. The first attempts yielded meager results. More recently Porter (1956) and Selby (1955) have given excellent accounts of certain aspects of the basal layers and the adjacent dermis. Our work on the hair follicle has just appeared (Birbeck and Mercer, 1957)

II. The Basal Layer Cells and the Dermal-Epidermal Junction

Beneath a keratinized epidermal tissue we can always distinguish a dermal-epidermal junction, a boundary, which separates a community of cells, whose characteristic product is the extracellular, fibrous protein collagen, from another more closely knit community, the epidermis, producing typically, keratin Here one finds, in each instance, germinal cells attached to the dermal-epidermal junction, and cell streams moving outward from the junction exhibiting an orderly sequence of differentiation

The cells of the basal layer are usually columnar and appear to be attached at one end to the dermal-epidermal membrane The situation is essentially the same in skin, hair, and feathers (Figs. 1, 2, and 3) The dermal-epidermal junction, to the electron microscopist, consists of a continuous structureless, extracellular membrane (m) distinct and separated from the plasma membranes of the basal cells which are fastened to it by a cementing layer of poor electron scattering power (Porter, 1956, Selby, 1955, Ottoson *et al*, 1953) In skin, numerous fine extracellular striated fibrils (CF) are closely applied to the membrane on the dermal side (Porter, 1956, Weiss and Ferris, 1954) It is a matter

of definition whether part of this fibrillar layer is included in a "basement" membrane. Porter's pictures of the skin of a newt larva (Porter, 1956) give a more definite impression of a discrete, well-attached fibrous layer than one finds in mammalian skin. In the hair and feather follicles the basal layers abut on a papilla containing blood vessels with their associated membranes and a variety of cell types.

The basal cells themselves have very similar characteristics in all epidermal tissues. Although fastened to the basal membrane, they show little attachment to each other and great surface activity manifested by many fine fingerlike projections (Selby, 1955). Their cytoplasm is densely populated with fine dense granules of the type which Palade (1955) has given reasons to regard as "ribonucleoprotein" particles (RNP). Neither at this level, nor farther from the basal layer, are these particles associated with a system of membranes to form an endoplasmic reticulum. This situation—large numbers of free RNP particles (strong basophilia) and an absence of a reticulum—we take to be a feature of well-differentiated cells which have a great potentiality for protein synthesis but no machinery for secretion. The epidermal cells are capable only of a single burst of synthetic activity which fills their confines and brings their life to an end Undifferentiated embryonic cells also have many free RNP particles, but in this case cell division accompanies synthesis which is not yet specialized toward the mass production of single products.

III. Cell Membranes and Differentiation

An important structure not directly visible in the light microscope, but which is readily visible in the electron microscope, is the cell membrane or plasma membrane A striking feature of the basal cells and of the cells lying immediately above them is their very convoluted plasma membranes and limited areas of close contact. We take these features to be evidence of poor *intercellular adhesion* and of a *related mobility* of the unattached membranes. It may seem a matter of logic as well as observation, that if cells are to move, grow, and divide, they must have a measure of independence. On the other hand, as units of a stabilized cellular community, they must stick together This is, in fact, what we observe. Unstabilized, free cells have irregular convoluted surfaces The common form of contact in an established tissue is a broad smooth area of membranes separated by a rather constant spacing of the order of 100–200 A which appears to be maintained by an intercellular cement of low density.

The epidermis offers us an admirable opportunity to observe the establishment of a tissue, since the whole story from division to dead

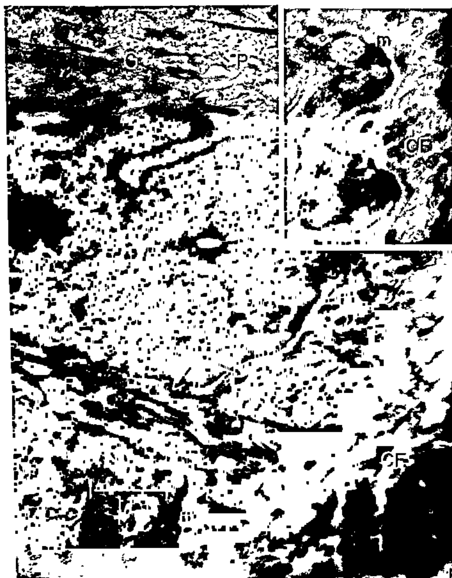


FIG 1 Vertical section through the thick skin of a rat's paw showing the dermal-epidermal junction and the basal layer cells Longitudinal and cross sections of collagen fibrils (CF) appear in the dermis on the lower right, the thick membrane of the junction appears at m N, nuclei, P, groups of RNP particles, M, mitochondria At F small bundles of keratin fibrils (tonofibrils) can be seen Notice the irregular membranes of the Malpighian cells at G Cell contacts appear at T INSET An enlargement of a portion of Fig 1 showing the detail of the basal membrane (m) Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

and hardened tissue is preserved at one time The hair root yields perhaps the most information, since as many as six-cell streams form from the same germinal matrix, each with a distinctly different course of differentiation, shown by the time of appearance and type of intracellular product.

While the causes of these events remain obscure, we have been able to observe some differences in the way in which the cells in the several streams settle down and establish neighborly relations (Birbeck and Mercer, 1956). The presumptive cuticle cells lead the way in these changes, followed closely by the cells of the inner root sheath. Contacts in the germinal matrix are not conspicuous and are limited to small local patches (Fig. 4); but the cuticle cells soon become recognizable by reason of a thickening of their membranes and an increase in the area of close cell contact. It is as if the cell surfaces were becoming sticky and, in a zipperlike fashion, were effacing the intercellular gaps by the spreading of the area of contact (Figs. 4 and 8). The membranes of the cells of the inner root sheath also become closely opposed but remain less conspicuous than those of the cuticle. In contrast, the presumptive cortical cells retain their surface activity; contact is local with many intercellular gaps which are occupied by the processes of the melanocytes if the hair is pigmented. The taking up of pigment by the cortex may thus be seen as a consequence of continued membrane activity (Birbeck and Mercer, 1956).

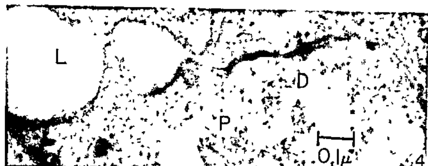
While these changes may be seen as early events in the progress of differentiation, they are not primary and we are still faced with the problem of why the cuticle leads the way Perhaps its position relative to the outer root sheath and the dermal layers is crucial, but we can offer no electron micoscopic evidence bearing on the point.

The early cell contacts are characterized by a spacing between the plasma membranes of the order of 150–200 A (Fig. 4) This type of

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FIG 2 Section through germinal layer of human hair follicles, showing the papillary membrane and several germinal cells In the papilla (Pap) may be seen various cells and membranes derived from the dermis M, mitochondrion, T, cell contacts, m, membranes Material fixed in buffered osmium tetroxide and embedded in an epoxide resin.

FIG 3 The germinal layer of the feather The papilla (Pap) contains numerous cells not belonging to the keratin-forming system which is separated from them by the membrane (m). Notice the lack of cell adhesion and the sections of small pseudopods between the Malpighian cells (G) N, nucleus, P, group of RNP particles Material fixed in buffered osmium tetroxide and embedded in an epoxide resin


L
D
P
0.1μ
4


T
T
0.1μ
5


0.1μ
K
6


0.1μ

spacing is found in most tissues and might be referred to as normal. Higher in the hair follicle the intercellular gaps widen to about 400 A with a further deposition of material between the membranes In the hair cuticle and in the sheath, several intercellular sheets may be found (Figs. 6 and 7) (Birbeck and Mercer, 1956). The nature and function

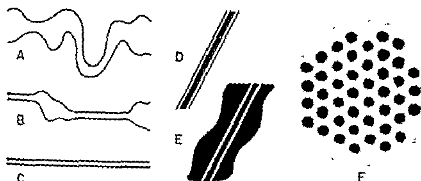


Fig 8. Varieties of cell membranes and contacts found in epidermal cells A. Convoluted unattached membranes in the basal layers B Localized contacts found particularly in skin, outer root sheath, and feather Characterized by a localized thickening of the membranes due to an intercellular deposit and an intracellular deposit Tufts of fibrils may be found extending intracellularly from these contacts C Cemented condition normal in stabilized tissues (spacing 150–200 A) D and E Elaborations of intercellular deposits (cements) found in keratinized layers F Proposed model of fibrous keratin The fibrous component, α-filaments (diameter about 60 A), is cemented together by an amorphous component γ containing more cystine than the filaments

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Fig 4 Cell contact near middle of hair follicle showing unattached convoluted areas (L) and areas with denser plasma membranes and intercellular deposits (cement) which have formed definite contacts (D) P, group of RNP particles Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

Fig 5 Plate type contacts (T) between feather cells characterized by dense membranes over limited areas, intercellular deposits cements, and within the cells a gathering of dense material Such contacts also occur in skin and the outer root sheath Stained with phosphotungstic acid (PTA)

Fig 6 Cell contacts in cuticle cells of hair Keratin deposits appear at K (amorphous in cuticle cells) The plasma membranes have opened to a distance ~ 400 A and several sheets have appeared between them Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

Fig 7. Contacts between Henle layer cells of the hair A single dense layer occurs midway in the cement uniting the cells Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

of these extra deposits is not known, but they may contribute toward stabilizing the membranes against keratinolytic solvents (Mercer, 1953)

A comment may be added concerning "nodes" and "intercellular bridges" (Porter, 1956). These structures appear to be deduced from the light microscopic images of the formations, seen in greater detail at N in Figs. 1, 5, and 12 These are local contact patches, with thickened membranes, backed by amorphous intracellular deposits and sometimes centers from which tufts of fibrils spread out into the cytoplasm. There is no continuity of the fibrils through the contact.

IV. Cell Synthesis

In the hair root we find several cell lines which specialize in the production of different products in a state of comparative purity. the cortical cells produce fibrous keratin, the cuticle cells a peculiar form of amorphous keratin; and the inner root sheath cells trichohyalin, a *variety of keratohyalin. All these cells have similar cytoplasmic and* nuclear equipment to begin with· an "active" looking nucleus, with a well-marked nucleolus and RNP particles immediately beneath the nuclear membrane, a cytoplasm well-stocked with RNP particles, a variety of agranular vesicles (Golgi type), and a poor development of endoplasmic reticulum There seems nothing in this equipment which we can seize upon to account for the different substances which soon appear. The only original difference seems to lie in their distance from the papilla and outer root sheath, but how this variable operates is still speculative

A. *Trichohyalin*

The first product to appear is *trichohyalin* in the cells of Henle, the outermost layer of the inner root sheath This substance appears as small amorphous droplets, TR (Fig 9), without a retaining membrane, which rapidly aggregate to form larger droplets At about the level of the constriction of the follicle, at a point easily recognized in the light microscope, particularly between crossed polaroids, the trichohyalin droplets suddenly transform into a fibrous birefringent modification These cells can be pin pointed in the electron microscope and

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Fig 9 The point of transformation of the trichohyalin droplets (TR) into fibrils (F) in the cells of Henle in the inner root sheath of the hair (PTA stained)

Fig 10 Early formations of keratin fibrils in the cortical cells of human hair At f is seen a few individual filaments, the larger masses (F) would be visible in the light microscope as fibrils Many RNP particles are present (P) Material fixed in buffered osmium tetroxide and embedded in methacrylate


F
f

interesting photographs of this astonishing intracellular transformation from the amorphous to fibrous form can be obtained (Fig 9). The fine fibrils, F, are seen to be moving out in a direction parallel to the axis of the hair root from each end of lenticular-shaped droplets. Cross sections of the transformed trichohyalin are difficult to interpret. They could be either sections of closely associated filaments or of crumpled sheets. The fibrous form is not drawn out of the droplets by cell extension because the elongation is far greater than could be produced by the lengthening of the cell. A better explanation would be that the fibrous form is "crystallizing" out at the surface of the droplets and moving away as further crystallization occurs. The longitudinal orientation is probably initiated by the flow of the cell contents. Unfortunately little is known chemically of trichohyalin but it appears distinct from the fibrous keratin of the cortex (Montagna, 1956, Rothman, 1954).

B. *Fibrous Keratin*

Fibrous keratin is first detectable in the cortex as wispy clumps of fine filaments, a little above the widest portion of the bulb (f, ca. 60–100 A) (Fig. 10). On first appearance these filaments are already oriented parallel to the axis of the hair. The long mitochondria are also oriented Since the cells here are already elongated by their movement toward the follicular constriction, we may suspect again that the slight shear of the cell contents due to flow is responsible for initiating the orientation

As far as we can see there is no precursor to the filaments of keratin It may be present in too small quantities or be of too low molecular weight for current microscopy. In any case, there is a striking contrast between the formation of the two fibrous products—keratin and trichohyalin (cf Figs. 9 and 10).

FIG 11 Cross section of human hair follicle showing the edge of three flattened cuticle cells and portion of the inner root sheath Mid-keratinization zone At m are cell membranes of type shown in Fig 6, M' of type in Fig 7 (inner root sheath) The keratin forms dense lumps (K) packed against the outer cell membrane in each cuticle cell The rest of the cell is occupied by the cell apparatus, nucleus (not visible here), RNP particles (P), and vesicles (Golgi) Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

FIG 12. Portion of a cell forming fibrils of feather keratin (domestic chicken) This cell is typical of cells forming fibrous keratin The nucleus (N) shows a nucleolus and a double membrane, mitochondria appear at M A cell boundary crosses the picture from the middle bottom to the top right hand corner Two cell contacts of type shown in Figs 5 and 8B appear at T Clumps of keratin fibrils are seen at F P, group of RNP particles Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

In the mid and upper bulb the cortical cells also receive their pigment from the melanocytes stationed a little below the tip of the papilla. This will be discussed in Chapter 12 of this volume.

Synthesis is more delayed in the cuticle cells. These cells are deformed into their familiar overlapping condition in the upper bulb and it is not until near the level of the constriction that droplets of a peculiar amorphous type of keratin, K, appear in small clusters and move toward the outer membrane of each cell (Fig. 11). The packing of these droplets into a layer against the membrane is responsible for the lamellar structure of the cuticle cells (Birbeck and Mercer, 1957).

The physical difference between the fibrous keratin of the cortex and the sheet of essentially amorphous keratin of the cuticle is responsible for many of the differences in the properties of these cells.

Turning to feathers (Fig. 12) and skin (Fig. 1) we find situations essentially similar to that found in the cortical layers of hair. Parallelism of fibrils is less apparent in feather cells (Fig 12) and in skin, many filaments seem to form tufts running away from the areas of close intercellular contact (Fig. 1) The same complex of mitochondria, RNP particles, and agranular vesicles characterizes the cytoplasm

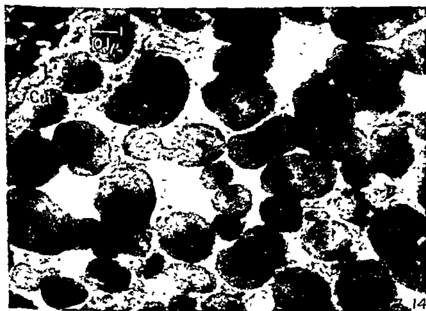
V Keratinization in Hair

In the cortical cells of the hair, the *filaments* of keratin rapidly accumulate in the upper levels of the bulb and cluster to form sheaves which become visible in the light microscope as *fibrils* These are most clearly seen in cross sections (Fig 13). In the "prekeratinous zone" immediately following the constriction of the follicle, these fibrils become denser and more discrete A certain amount of lateral fusion of the fibrils occurs and increases as the fiber becomes more fully keratinized.

In cross sections, in the prekeratinous zone the individual filaments within the fibrils can still be made out as light dots on a darker ground (Fig 15) In other words the filaments now appear to be embedded in a material denser than themselves Since this specimen has been fixed in an osmium tetroxide solution, the density observed is due in part to

Fig 13 Cross section of the cortex of the human hair at the middle of keratinization zone showing the accumulation of fibrils (F) before they have begun to fuse Irregular lines of cell membranes (m) can be found crossing the field (G), pigment granules N, nucleus (PTA stained) Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

Fig 14 Portion of cortical cell showing the "whorl" structure of the fibrils "Cut" is the edge of the cuticle Material fixed in buffered osmium tetroxide and embedded in an epoxide resin


Cut
14

level of the keratohyalin transformation, there is a very sudden rise in birefringence and its orientation is more nearly parallel to the skin surface in contrast to that of the lower layers, where it is dispersed but on the average at right angles to the surface

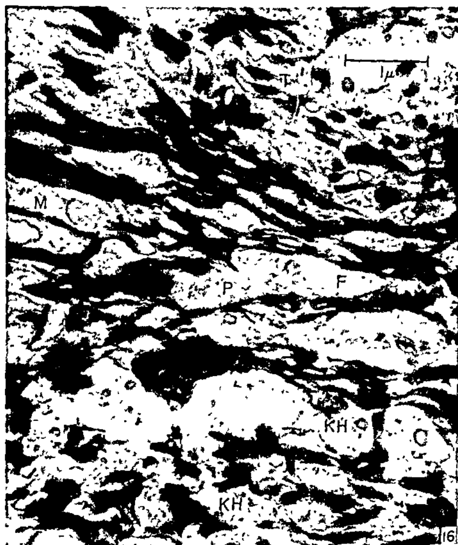


FIG 16 Portion of a vertical section through the upper layers of the epidermis of the plantar skin of a rat showing keratohyalin granules (KH) transforming into fibrils and other fibrils (F) of presumably keratin Cell membranes and complex contacts appear at T (see also Fig 5) M, mitochondrion, P, group of RNP particles Material fixed in buffered osmium tetroxide and embedded in an epoxide resin

With such evidence we could make a case for the horny component of skin being a mixture of fibrous keratin (hair cortex type) and fibrous keratohyalin This is not too far from opinions held earlier by certain histologists. We could perhaps try to check such a view if reliable analytical data of the several layers of skin were available, but the position is not hopeful for such an attempt. The difficulties in the way of obtaining and interpreting such data have been discussed recently by Rudall (1956) and by Ward and Lundgren (1954).

VII. Summary

1. Electron microscopy of thin sections of hair and feather follicles and of skin provides a picture of the developing cells of these tissues Direct comparison with light micrographs permits an unambiguous identification of structures

2 In each case a dermal-epidermal junction can be found consisting of a single continuous membrane (200–300 A thick), distinct from the plasma membranes of the basal layer cells, and separated from them by a layer (ca. 200 A thick) of material of low electron density which may represent a cement layer

3 The basal layer cells, columnar in the hard keratins and less markedly so in skin, contain an abundance of dense particles, presumed to be ribonucleoprotein particles, numerous mitochondria, a few agranular vesicles, and no organized endoplasmic reticulum This situation is thought to characterize cells with a great potential for protein synthesis but without the means for secretion extracellularly

4. The cell membranes of the basal cells and those immediately above them are extremely convoluted, giving the impression of a poor degree of intercellular adhesion and great surface activity This condition is interpreted as a characteristic of undifferentiated cells in an unstabilized tissue.

5. Differentiation seems to be initiated by an increase in intercellular adhesion incurred as a result of the appearance of an intercellular "cement" of low electron density This development can be followed most clearly in the hair root, where the course of differentiation is different in the several cell streams leaving the undifferentiated zone of the follicle to form the several layers of the follicle

The intercellular deposits uniting the membranes become more conspicuous higher in the follicle as a result of the widening of the intercellular spaces and the deposition in them of denser layers of material

6. The course of synthesis of fibrous keratin in the hair cortex, of amorphous keratin in the cuticle, and of trichohyalin in the inner root

CHAPTER 6

Electron Microscopy and the Biosynthesis of Fibers

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I INTRODUCTION

Biological fibers are more or less regular aggregates of macromolecules, whose dimensions may extend from the molecular into the histological level and beyond. When we consider specifically the synthesis of such systems, we feel impelled, over and above the primary biochemical problems, to pay special attention to the supermolecular aspects and to give an account of the genesis of these large structures For this reason microscopy, in its various forms, plays a much larger part in our investigations than is the case with the smaller, soluble molecules found in living systems

In the formation of many natural fibers, we may distinguish two main steps which can be investigated separately and by different techniques. The first, which is more properly the *synthesis* in the biochemical sense, is the formation of a high molecular weight, nonfibrous precursor. This step is most conveniently studied by conventional biochemical techniques We are probably on safe ground in assuming that the findings of biochemists concerning the synthesis of macromolecules are applicable to the special class of macromolecules destined to form fibers One of the objects of this article is to show that the cytology of fiber-forming cells is sufficiently like that of other synthesizing cells to suggest that the early stages are the same in all cases Unfortunately,

finality has not emerged as yet concerning the biochemical steps in the synthesis of macromolecules and little would be gained here by trying to summarize a perplexing situation (Simkin and Work, 1957).

The second step is the conversion or *transformation* of the precursor into the fibrous form. Here, with the appearance of units of a suitable size, electron microscopy begins to play an essential part

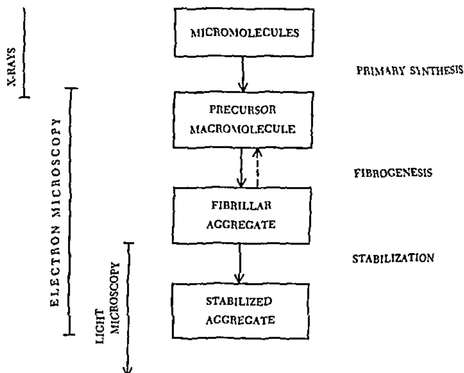


FIG. 1 Steps in the synthesis of fibers as explained in the text On the left hand side, the range of the various instruments is indicated

Probably not all fibrous systems will conform to this scheme and too little is known to be lavish with generalizations In at least one example, fibrous keratin, a precursor has not actually been found by electron microscopy

When two stages occur, the first always takes place within the living cell. The second may occur either inside the cell or outside of it—even *in vitro* Not only is the living cell unnecessary, but no product of it, such as an enzyme, may be required Naturally, this step can be more easily studied and is better understood It is a great advance when a precursor can be isolated—either from the formative cells or from the

fiber itself by reversing the process of transformation—and its *in vitro* behavior studied. When it also proves possible to cause the precursor to form fibers *in vitro*, we have the opportunity to study this process in detail With appropriate mechanical or other orienting influences, fibrous masses with a structure apparently identical with some natural formations may be produced It is a further problem to identify such influences in the natural state.

It will be advantageous to separate the process of fiber formation into several steps which have been set out diagrammatically in Fig 1. The scheme is purely formal and the occurrence of any step in the formation of a particular fiber can be decided only by observation The first step, already described above, we shall refer to as the *primary synthesis* The actual process of transforming the precursor into a material having a fibrous texture will be called *fibrogenesis*. By *organization* is meant the stages following fibrogenesis in which the fibrous material is built into larger sized units. *Stabilization* (tanning, keratinization, etc.) is a process which may follow organization and convert the fibers into a more durable material

The central importance of the electron microscope in the study of the structure and formation of fibers arises from the fact that it is uniquely suited to study objects with dimensions in the range 20–1000 A and most of the structures we are concerned with macromolecules, protofibrils and their early aggregations, have sizes included in this range

II The Fine Structure of Fiber-Forming Cells

Current research into the nature of biological synthesis is characterized by the attempt to relate the visible structural elements of the cell and the underlying biochemical activity. The prevailing opinion is that the nucleic acids are somehow involved in protein synthesis In particular, the ribonucleoproteins (RNP) of the cytoplasm, which are found in the microsome fraction when homogenized tissues are fractionated, are closely coupled with synthesis (Simkin and Work, 1957) Since microsomes are beyond resolution in the light microscope, special efforts have been made with the use of electron microscopy to discover the structures corresponding to microsomes in the original cell. This intensive study, associated particularly with the names of Palade, Porter, Sjostrand, and Bernard, has now established the main features of the fine structure of cells which synthesize protein Palade and Siekevitz (1956) have summarized the reasons for believing that the microsomes result from the breakdown of an elaborate system of particle-studded membranes in the original cell, which was named the "endoplasmic

reticulum" by Porter (1953). Palade (1955) has identified the dense particles (100–300 A diameter) attached to the membranes with the RNP of the cell. The function of the membranes, or for that matter their exact morphology, is not fully understood. They, unlike the particles, are not necessary for synthesis since they are absent from epidermal keratin-forming cells (Mercer, this volume, Chapter 5), but they may be a device for facilitating the orderly transport of metabolites, raw materials, and products which build up in a cell having a prolonged life as a protein former. The dense particles are associated with the basophilia demonstrated histochemically and earlier related to synthesis (Brachet, 1947).

A further characteristic of active cells is a large basophilic nucleolus and basophilic deposits at the nuclear membrane. In the electron microscope the nucleolus is equally well marked and contains particles apparently identical with the dense particles of the cytoplasm. These same particles often gather inside the nuclear membrane. This membrane is double and the inner layer appears to have pores Watson (1954) has shown micrographs to suggest that the RNP particles pass through the pores and, becoming associated with the outer membrane, form part of the endoplasmic reticulum

Most secretory cells also have vesicles, often elongated, whose membranes are free of RNP particles. When these cluster in a small compact group, they are identified with the Golgi apparatus of classic histology. It cannot be said that electron microscopy has helped much in clearing up the mystery of its function.

This work was carried out mostly on liver and certain glands and little has been done on cell systems whose products are the precursors of fibers To see whether the generalizations outlined above have a wider application, the following cells, making well characterized fibers, have been studied.

1. The silk-producing cells of the silkworm (*Bombyx*) The fame of the silkworm as a protein factory is well based and one might reasonably expect to find an hypertrophy of the synthetic apparatus in the cells of the silk gland

2 The cells of the left colleterial gland of the female cockroach (*Periplaneta*) which produce the protein of the egg case Interest in egg case material is due to Rudall, who has shown, at least in the case of a mantid, that it yields a remarkably detailed α-type x-ray pattern (Rudall, 1957). The α-type x-ray patterns are given by many proteins of which the type example is mammalian hair keratin (Astbury, 1947).

3. *Certain chitin-, as distinct from protein, producing* cells of the

silkworm and cockroach Chitin is an example of a fibrous polysaccharide found either as well oriented fibers or as membranes made from fine fibrils, e g , the peritrophic membrane secreted by the mid-gut cells (Mercer and Day, 1952).

4. Collagen-forming cells (fibroblasts).

5. To these we may add, for comparison, the epidermal cells of the skin already described in a previous article (this volume, Chapter 5).

There are, of course, great numbers of other cells in which fibers are found, either as special products or as part of their normal structural equipment, which could have been examined However, the cells chosen will illustrate the formation of an example of each of the three main classes into which Astbury has divided fibers (the α, β, and collagen types) and also will include intracellular and extracellular fibers.

III Silk-Forming Cells of the Silkworm

Silk is synthesized as a soluble precursor by the silk glands of many caterpillars and stored in a dilated portion of the gland, the storage vessel In the common silkworm, two different proteins are formed· *fibroin*, the silk thread proper, and *sericin* which glues the threads of the cocoon together. The fibroin is formed mainly by the cells of the more distal portions of the gland, which were those examined The gland has a structure of diagrammatic simplicity. The walls are a unicellular layer of cells, the raw materials of synthesis diffuse in through the outer surface and the protein produced passes through the inner face into the lumen of the gland The cells are thin but very large in other respects, being visible to the unaided eye The nuclei are extraordinarily large and arborescent in the mature larva

Electron microscopically (Fig 2), by the criteria described above, the picture is one of intense activity. The nucleus reveals a multitude of nucleolarlike bodies, the cytoplasm is packed with dense granules (P) arranged in striking patterns with many mitochondria (M) scarcely visible in the crowded array. The membranes, to which the particles are attached, are not readily made out in osmium-fixed material such as shown in Fig 2. They can, however, be shown more clearly if potassium permanganate is used as the fixative (Luft, 1956). This fixative, a valuable new addition to the electron microscopist's armory, destroys the RNP particles and reveals the membranes in their full clarity (R) (Fig 3). In other pictures the fibroin (F) may be seen moving toward and gathering in lumps against the internal membrane, which is often cast into long fingerlike projections

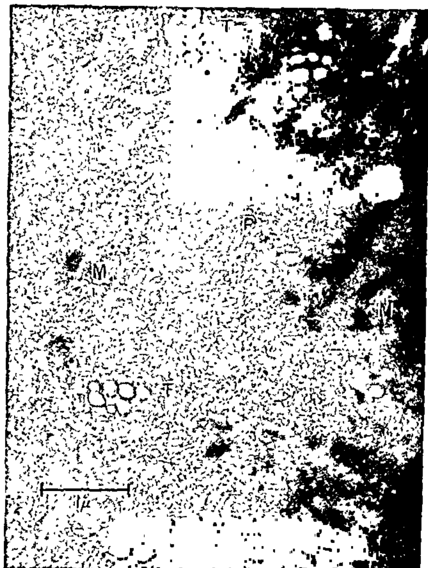


FIG 2 Portion of cell from the silk glands of the silkworm. The caterpillar was fully grown and about to cease eating prior to commencing spinning The cell shown was in the distal portion of the silk gland where, it is thought, fibroin is the main product. The whole cell is closely packed with particle-studded membranes forming a complex reticulum Nearer to the inner face of the cell (not shown here) the silk collects in large amorphous lumps M, a mitochondrion, P, dense particles of RNP, T, sections of fine tracheoles [Fixed in buffered osmium tetroxide solution (Porter, 1953, Palade, 1955) and embedded in an epoxide resin (Mercer, this volume, Chapter 5)]

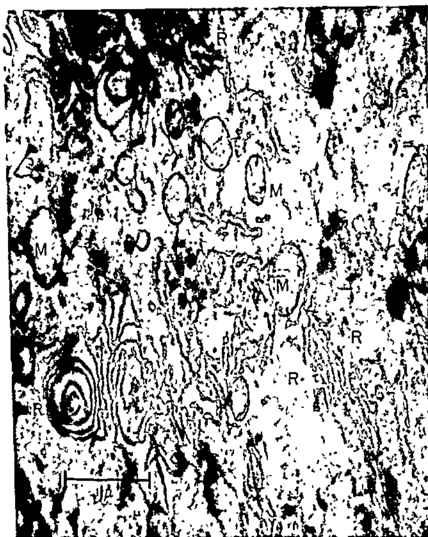


FIG 3 Portion of silk-forming cell of the silkworm, fixed in buffered (7.2) potassium permanganate (2%) (1 hour at 5°C) to be compared with Fig 2, which is of material fixed in osmium tetroxide solution The RNP particles have been destroyed but their supporting membranes (R) are preserved and clearly seen in contrast to Fig 2. Mitochondria (M) have their external membranes preserved but the internal structure is damaged indicating a different composition

IV. The Left Colleterial Gland

Cytologically the left colleterial gland resembles the silk gland It consists of an aborescent clump of tubules, *each a monolayer of cells*, enclosing a lumen containing secretion Internally, another group of cells secretes a chitinous cuticle (L) which lines the lumen and opens



Fig 4 Secreting portion ("end apparatus") of a protein-producing cell of the left colleterial gland of the cockroach Closely packed particle-studded membranes of the reticulum are seen at R almost filling the entire cell The nucleus is beyond the left hand end of the cell The secreting membrane is seen extended into long fingers (F) and drops of secretion (S) extending beyond the fingers are seen moving out of the cell to collect in the lumen to the right Portions of two chitin-producing cells (C) seen at each side of the cell opening, produce the dense chitinous cuticle (L) which lines the lumen and opens into each protein-producing cell to form the peculiar funnel-like formation The distended plasma membrane of the cell plunges into the "funnel" but is everywhere continuous although greatly convoluted Many mitochondria (M) and agranular vesicles (V) are also to be seen [Fixed in buffered osmium tetroxide solution (Porter, 1953, Palade, 1955) and embedded in an epoxide resin (Mercer, this volume, Chapter 5)]

into each cell to form a funnel complete with turned back edges into which the cells pour their secretion (S) (Fig 4). This structure is referred to as the "end apparatus."

The endoplasmic reticulum (R) is again extraordinarily well developed and the nucleus possesses a large granular nucleolus. The chitin-forming cells have no reticulum and few RNP particles.

The plasma membrane extends in long fingerlike processes into the funnel and the secretion (S) is seen in droplets separating from the end of the fingers The droplets are not vacuolated at this time.

V. Chitin-Forming Cells

Chitin-forming cells are found in many sites in insects Those in the colleterial gland have been mentioned Others secrete an external membrane to organs, and also form the tracheoles The mid-gut cells secrete the peritrophic membrane, which will be discussed later.

All these cells have active nuclei, very many mitochondria and vacuoles, but few RNP particles Thus, the nucleic acids and endoplasmic reticulum seem to be associated with protein synthesis and not with carbohydrate synthesis; as is already known from biochemical studies, starch can be formed enzymatically *in vitro* in the absence of nucleic acid. The large development of α-granular vesicles may represent a reticulum without RNP particles and concerned with secretion.

VI. Collagen-Forming Cells

Collagen is made in cells found scattered about in connective and dermal tissue, and are often referred to as fibroblasts. The cross-striated fibrils of collagen form extracellularly and a precursor, "procollagen," an elongated particle, is supposed to be secreted by the cells (Gross, 1956).

The fine structure of certain fibroblasts has been described (Jackson, 1955, 1956). They contain an endoplasmic reticulum (R) and a special kind of small vesicle (V) that may be seen in Fig 5, which shows a portion of a cell from rat skin. Fine threadlike particles also occur, particularly adjacent to the nucleus, somewhat similar formations may be sometimes seen outside the plasma membrane (M).

VII Epidermal Proteins (Skin, Hair, and Feather)

The cytology of the cells of the epidermis has been discussed in another article in this volume (Mercer, Chapter 5) and only certain features will be mentioned here for purposes of comparison Both keratin and keratohyalin (or trichohyalin) remain within the epidermal cells. In epidermis, two types of proteins are produced in the

same cell but in the hair follicle they occur in separate sublines of cells, each derived from the matrix. Keratohyalin is an example of a fibrous substance formed as an amorphous precursor—the familiar keratohyalin droplets of histology. Keratin does not seem to have a precursor or it occurs in too small quantities to be seen.

Epidermal cells are basophilic and contain large numbers of RNP particles, but these are not associated with membranes to form an endoplasmic reticulum although many α-granular vesicles are present We



FIG 5 Cytoplasm of a collagen-producing cell ("fibroblast") from the dermis of the rat The cell was surrounded by mature collagen fibrils recognized by their characteristic transverse striations (CF) The nucleus is not shown The particle-studded membranes (R) forming the reticulum are very open and may form a continuous system leading to the surface (e g, at S) A characteristic feature of collagen-forming cells is the numerous small vesicles (V) which are not apparently part of the endoplasmic reticulum M is the cell membrane [Fixed in buffered osmium tetroxide solution (Porter, 1953, Palade, 1955) and embedded in an epoxide resin (Mercer, this volume, Chapter 5)]

believe these features to be characteristic of cells which retain their products within them (Mercer, this volume, Chapter 5)

Summarizing these findings, the cells forming protein-fiber precursors have essentially the same fine structures as other cells secreting soluble proteins and hence presumably the same biochemical processes occur. However, if the cells retain their products, an endoplasmic reticulum may not form, although RNP particles are present. Chitin-

forming cells have no granular reticulum and few RNP particles free in the cytoplasm, but have many agranular vacuoles near the secreting surface of the cells.

VIII. From Precursor to Fiber

In this section we shall discuss fibrogenesis and organization, the steps following synthesis, indicated in Fig. 1.

A. *Fibrogenesis*

When we consider this change, we must bear in mind that there is no reason to think that we are dealing with a single universal process. The end result, the appearance of a material having a fibrous texture from a nonfibrous precursor, may be much the same, but may be reached by different routes For example, F-actin[1] may be produced by a linear aggregation of large corpuscles (Szent-Gyorgyi, 1947); on the other hand, the female mantid, when making the raw material for the egg case, prefers to "blow" bubbles and pull these into ribbons (Rudall, 1957). In short we have to consider each as a special case and investigate it separately.

Several fibrous systems seem to have as an elementary building unit a long thin filament, which can be called a "protofibril." This filament seems to form directly from the precursor Its lateral dimensions are usually greater than those of a single molecular chain (10–20 A), so that we must assume that it has an inner structure made up of several chain molecules. In a theoretical sense this could be formed. (a) by the aggregation of single molecular chains produced by the unfolding of precursor molecules (Fig 6A) *or* (b) by the aggregation of precursor molecules without unfolding (Fig. 6B). This phenomenon resembles crystallization.

The unfolding and spinning hypothesis, naturally comes first to mind because it is the theory behind the manufacture of artificial fibers The silkworm is supposed to form silk in this way but, as we shall see, there are complications in this simple view. When we consider some of the ways in which fibers appear in biological systems, it is not so easy to suppose that they are produced by spinning and drawing based on unfolding Fibril formation in a solution of the precursor can be initiated by a slight change of variables and can be reversed by an equally small change. The fibrils may separate from a solution containing other macromolecules, much as a pure crystal may separate from a mixed solution A mechanical factor, which could be thought to "draw the

[1] F = Fibrous form.

fibrils out," is often lacking. Further, the resulting filaments may have a constant width and a neat appearance, not at all like the "fringed micelle" to be expected from the aggregation of long chains. The x-ray analysis of many fibers reveals an elaborate inner structure, suggesting an ordered arrangement from macromolecular to almost atomic dimensions and resembling that of a protein crystal (Astbury, 1947).

Fibrils can be formed from insulin, a protein which, owing to the cross-linking of its four chains, cannot unfold completely. Figure 7, for example, shows some filaments of F-insulin. X-ray analysis reveals a well-ordered structure (Bear, 1952). The biologically active insulin can be recovered by reversing the fiber-forming process.

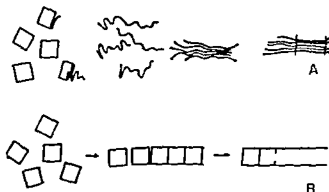


FIG 6 Two methods of forming protofibrils from a precursor molecule of the corpuscular type A. Unfolding The precursor molecule is supposed to unfold into polypeptide chains which aggregate as fibrils (crystallites) in which the molecules lie parallel in the β-type configuration B Linear aggregation of corpuscular molecules without unfolding A variation of this type of fiber formation (helical aggregation) is shown in Fig. 8

Hypothesis (b) avoids most difficulties since it supposes that the precursor molecules remain intact when incorporated into the protofibril, which is then more properly regarded as a variety of long thin crystal

An aggregation of large units has been suggested in the case of F-actin (Szent-Gyorgyi, 1947) and F-insulin (Pauling, 1953) (Fig 7), both of which are found in the form of long filaments of very constant diameter, capable of being reconverted into the soluble form The width of the filaments is much greater than the diameter of the molecule of the precursor form, so it is clear that we are not concerned with a simple linear aggregate of the parent molecules. It is possible that the first step is the formation of a larger symmetrical corpuscle, which is the

unit that later undergoes linear aggregation (Szent-Gyorgyi, 1947). Alternatively, the actual structure may be a spiral (Fig. 8) which may give filaments of a variety of diameters (Pauling, 1953).

There is an impressive and growing list of such fibers with structural aspects most easily explained in terms of aggregation: collagen (Gross, 1956), keratin (Farrant *et al.*, 1947), feather keratin (Astbury and Marwick, 1932), fibroin (Mercer, 1951), fibrin (Hawn and Porter, 1947), trypsinogen (Gross, 1951), paramyosin (Hodge, 1952), and meromyosin (Philpott and Szent-Gyorgyi, 1954). Many of these can be obtained as fibrils or paracrystalline formations from solutions *in vitro*.

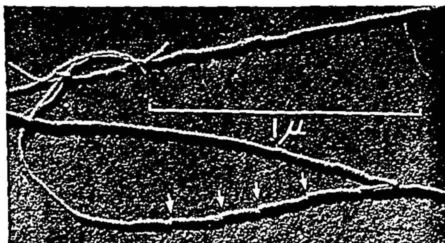


FIG 7 Electron micrograph of fibrils of F-insulin made by heating an acid solution of insulin. Shadowed with platinum The arrows mark breaks in the fibrils produced during drying and probably indicating planes of weakness at right angles to the axis of the fibril, which may be related to the inner structure Photograph taken from Farrant and Mercer (1952)

Unfortunately, no electron micrographs so far obtained have the resolution adequate to justify confident decisions The filaments must usually be shadowed with metal, and this precludes the possibility of observing small scale structures within them One is forced to place weight on the finding of discrete filaments of uniform diameter. Occasionally a regular nodulation or fracture suggests an inner periodicity (Fig 7)

Since some form of aggregation must occur in the formation of fibers, a number of writers have speculated concerning its mechanism. Opinions differ as to whether the forces causing aggregation should be con-

sidered specific or nonspecific. Rees (1951) has shown that, in certain conditions, a linear aggregate may be expected from the same nonspecific forces, that cause coagulation of colloidal suspensions. Barbu and Joly (1953) have found that fibrous aggregations can occur in solutions of proteins not normally regarded as fiber forming.

The aggregate, initiated by nonspecific forces, may later become consolidated by other forces effective at close contact. Some have supposed that there are certain "sticky patches" on the large molecules which are the seats of a specific attraction (Anderson, 1956). There is an important general principle, pointed out by Crane (1950), that a helical structure must result from the successive addition of asymmetrical elements (Fig 8) The linear aggregate is a degenerate case of

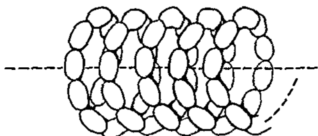


FIG 8 A helical aggregate formed by the continuous addition of unsymmetrical monomer particles The linear aggregate (Fig 6b) is the special case of the addition of symmetrical particles

the helix A helix also results if several different units participate. There is little doubt that Crane's principle should apply to the aggregation of macromolecules and, accordingly, that many of the single filaments we observe are helices

Silk fibroin should be given special note since it is usually cited as an example of unfolding We have earlier described the formation of the precursor which is stored in the dilated portion of the gland until needed An ultracentrifugal analysis of an extract of the gland reveals three components, but it is not known whether they are all associated with fiber formation. When an extract is allowed to stand, it grows turbid and more viscous and electron microscopy shows that long thin fibrils (ca 100 A diameter) have formed (Mercer, 1951, Kobayashi and Goto, 1956) (Fig 9) Assuming that the same events occur during spinning *in vivo*, it would seem that some kind of aggregation forming a fibril is also occurring here

B. Organization

Assuming that the basic fibril has been formed, we have now to consider the means by which this is used as a unit for the construction of higher ordered structures. The geometrical form of the structures is most easily discovered with the electron microscope, although it may be deducible from x-ray photographs or even with the light microscope.

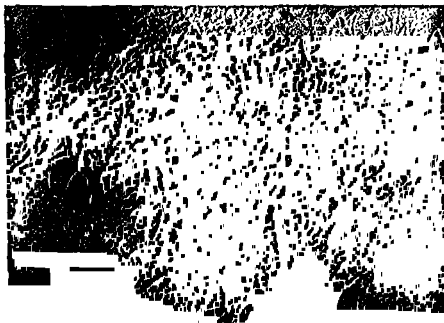


FIG 9 Electron micrograph of the fibrillar aggregate formed by allowing a solution of the contents of the silk gland of the silkworm to stand without disturbance. Well-formed fibrils (protofibrils) are formed but there is no orientation Shadowed with platinum

The problem is to find the factors, mechanical or otherwise, which organize it There is often a relation between the mechanical function of the fibrous system and its structure, which may provide clues

Some examples of the arrangement of the fine fibrils observed are shown in Fig 10 Tangled "brush heaps" (Fig 10A) arise in the absence of orienting influences, such as in a fibrin clot Fibers in which all the elementary filaments are parallel (Fig 10B) have been subjected to an orienting influence either during or after the formation of the filaments. The most obvious influence is the shear due to flow, which probably initiates the orientation of silk thread Drawing, after

extrusion, is also a factor in improving the orientation of silk, as it is in many artificial fibers.

The orienting factor is less obvious in the case of the keratin and trichohyalin fibers of the hair follicle. The cells in which these filaments form, are being slowly distorted into a spindle shape as synthesis proceeds, but the increase in length of the cell is not sufficient to draw out the filaments. In fact, the filaments *grow* parallel to the long axis of the cell. We are here forced to assume that the slight flow oriented the first formed filaments and that growth from these maintained the same orientation. Lateral association between neighboring fibrils probably also plays a part. In skin cells the fibrils lie parallel to the skin surface

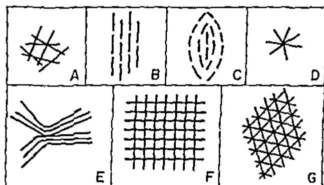


FIG 10 Various structures which may be constructed from rodlets and fibrils

but unoriented in other respects The orientation seems to be due to the flattening of the cells as they reach the upper layers of the epidermis The keratohyalin fibrils grow out of the droplets in a direction mostly parallel to the surface (Mercer, this volume, Chapter 5)

Tactoids (Fig 10C), familiar from their occurrence in tobacco mosaic virus (TMV) solutions, have been proposed as fiber-forming elements The rodlets of TMV are not unlike the protofibrils under consideration here and it seems quite probable that, in some instances, *e.g., the bundles of fine filaments of keratin in hair cells, the same* forces which maintain tactoids are operating. Unfortunately, physical chemists are still arguing about the nature of these forces (Pauling, 1953)

Spherulites and sheaves (Fig. 10D and E), e.g, in F-insulin (Far-

The filaments must also be supposed to possess [illegible]

Some of the more interesting structures are to be found among the fibrous membranes (Figs. 10F and G). Collagen in skin, and certain cuticles are often found arranged into parallel sets of fibrils forming approximately a right angle with a similar set above and below it (Reed and Rudall, 1948, Porter, 1956) The ultimate orienting influence is clearly the surface of the animal, which ensures that the whole formation lies parallel to it The immediate controlling influence is not so obvious. In other instances (Fig. 10G) the geometry is even more complex and sometimes of surprising regularity.

Such sheets may be formed by exfoliation from a surface composed of the cells that secrete the precursor. Here we can conceive of two kinds of organizer: (1) a pattern or "die" on the cell surface or (2) the existing pattern of the preceding sheet, which acts as a template for the assembly of its successor. The peritrophic membrane (Fig. 11) lining the mid-gut of insects could be an example of the first suggestion (Mercer and Day, 1952). The secreting cells are covered with projecting microvilli (the brush border of histology) with a cross-sectional diameter about the same size as the holes in the membrane. We could imagine the filaments forming in the grooves between the studs on the surface (Fig 12). Sections of the cell surface, although showing the pattern of microvilli and the layers of shed membrane, have not yet provided an example of a membrane in the act of formation, therefore decisive evidence is still wanting

The collagen meshworks in skin, and in earthworms seem to form some distance from the cell surfaces, which are covered with amorphous material (Porter, 1956). The "self-template" seems more likely here We can form a conception of how this could operate by supposing that the upper surfaces of fibrils have "studs" on them which fit into "holes" in other fibrils when these are laid across them at right angles.

This discussion of fibrogenesis and organization has been limited to the special case in which the elementary fibrous unit is a fine filament (or ribbon) While this is applicable to many systems, we must expect that other devices will be found. Rudall (1957), for example, has described fibrogenesis in the secretion of the colleterial gland of a mantid in which ribbons are produced by the elongation and flattening of vacuolated drops of the secretion. Further, some fibers appear directly as fibers. This occurs when the structure forms inside the cell. A distinction can be made here between fibers directly built from micromolecules, and those formed from macromolecules, which enter the fiber as soon as formed. We do not know if two such processes exist

C. *Stabilization*

This final process, which takes place in certain fibers and serves to harden them for special functions, is usually a chemical rather than a structural problem. The two forms of stabilization which have attracted most chemical interest have been the keratinization of hair, and the

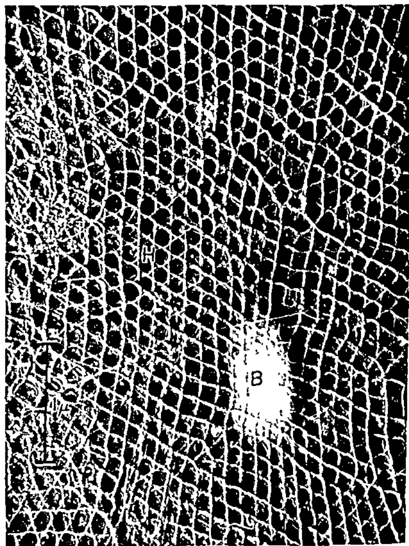


FIG 11 Electron micrograph of the peritrophic membrane of the cockroach Notice the well-formed hexagonal pattern in places (H) and defective structures elsewhere The large object is a bacterium (B) Photograph taken from Mercer and Day (1952)

tanning of the hard parts of insects. These chemical changes must take place after the final structure has been formed, and do not alter it. Fibrous keratin, for example, has much the same molecular structure before and after keratinization. We envisage the change occurring outside the "crystalline" filaments and not altering their internal structure

It is clear that little is known for certain about formation of these structures which are the direct products of living cells and which display, in their often elaborate organization, many attributes thought peculiar to living matter. It would seem, however, not beyond the immediate resources of an integrated biochemistry, physical chemistry, and electron microscopy to unravel the group of problems referred to

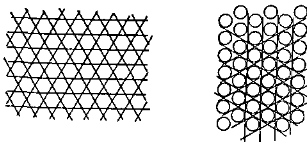


FIG 12. Drawing to show how a meshwork such as that in Fig 11 (see also Fig 10G) could be produced on a surface bearing a hexagonal pattern of "studs" as is found on the cells secreting the peritrophic membrane (Fig 11) in certain insects

above as "fibrogenesis" and "organization." The more difficult question, the primary synthesis of the macromolecular raw material, does not lack attention, but is likely to prove a tougher problem to solve.

IX. ACKNOWLEDGMENTS

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CHAPTER 7

The Chemistry of Keratinization[1]

A GEDEON MATOLTSY

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I GENERAL INTRODUCTION

A Biological Viewpoint

The term "keratinization" defines the activity of epithelial cells by which horny structures, such as hair, nail, stratum corneum, are produced From the *biological viewpoint* keratinization may be considered a specific differentiation process in which three kinds of epithelial cells are involved. undifferentiated cells, differentiating cells, and terminal cells (Hanson, 1947, Matoltsy and Sinesi, 1957, Montagna, 1956, Weiss, 1953)

The undifferentiated cells of keratinizing tissues are mitotically active, they produce cells of the same kind, and cells which enter the course of differentiation The keratinizing tissues are renewed by these

[1] The experimental work, described in this paper, was started in the Dermatological Research Laboratories, Harvard Medical School and Massachusetts General Hospital and was aided by grants from the American Cancer Society and National Institute of Health (RG-3921)

cells either continuously, as in nails and epidermis, or in cycles, as in hair.

The differentiating cells are active in (1) synthesis of prekeratin substances, (2) decomposition and elimination of nuclear and certain cytoplasmic elements, (3) release of water, and (4) coalescence of cell contents into a horny mass. Although differentiating cells of each keratinizing tissue show such activities, the actual mechanism of differentiation appears to be different in the various keratinizing tissues. This is suggested by the markedly differing morphological appearance of differentiating cells which may be illustrated by the following examples. The cytoplasm of differentiating medullary cells of the hair changes into a horny mass by a simple coalescence of components. Differentiating cortical cells of the hair show intense cytoplasmic fibrillation. In the cells of the inner root sheath of the hair follicle, trichohyalin granules appear. In epidermal cells cytoplasmic fibrillation and formation of keratohyalin granules can be seen. Keratinizing cells of the nail show cytoplasmic fibrillation without keratohyalin formation.

The terminal cells are metabolically inactive and consist mainly of keratin. The cortex of the hair, the nail, and the stratum corneum of the epidermis, all contain an α-type of keratin (Astbury and Woods, 1934, Derksen *et al.*, 1937, Giroud and Leblond, 1951, MacArthur, 1943, Rudall, 1947). The medulla of the hair contains a β-type of keratin (Stover, 1946, 1947, Ward and Lundgren, 1954) and the cuticle of the hair is amorphous (Mercer and Rees, 1946, Stoves, 1947).

B Chemical Viewpoint

From the *chemical viewpoint* keratinization appears as a most complicated process. Chemical complexity seems to reach its maximum in the prekeratinization and keratogenous regions where the production of prekeratin substances and the elimination of nonkeratin substances progresses simultaneously. In different keratinizing tissues such productive and resorptive activities are of different intensities. As a result, cornified cells are produced which contain keratin and nonkeratin substances in different ratios. For instance, while the mature cortical cells of the hair become filled almost entirely with keratin, and the medulla cells of the hair contain about 95% keratin (Matoltsy, 1953), the cornified cells of the epidermis contain only about 60 to 70% keratin (Matoltsy and Balsamo, 1955a, Spier and Pascher, 1953, Szakall, 1955). The nonkeratin components of cornified cells are nuclear and cytoplasmic remnants consisting of proteins, peptides, free amino acids, carbohydrates, lipids, etc (Matoltsy and Balsamo, 1955a, Rothman, 1954; Spier and Pascher, 1953, Szakall, 1955). Formation of keratin in various

keratinizing tissues seems to proceed according to different principles and consequently, chemically differing keratins are formed For instance, while hair, wool, and nail keratins have eminently high sulfur contents (Block, 1939, Wilkerson, 1934), *epidermal keratin contains about half as* much sulfur (Eckstein, 1935, Muting *et al*, 1955, Wilkerson and Tulane, 1939); the medullary keratin of the hair contains only a very small quantity of sulfur (Matoltsy, 1953; Stoves, 1947). Thus, generalizations regarding the mechanism of keratinization (Giroud and Leblond, 1951, Leblond, 1951) meet with various difficulties, and it is more appropriate to consider keratinization in each tissue as a separate mechanism.

In the subsequent discussions of keratinization only the hair cortex[2] and the epidermis of mammals will be considered. Chemical and physical aspects of keratinization will be related to morphological concepts, and cytodifferentiation of the hair cortex and epidermis will be briefly outlined. The characteristic features of cornified cells and the properties of their keratin component, as well as the characteristic features of differentiating cells and the properties of their prekeratin component will be presented A few mechanisms, which appear essential for keratin formation will be set forth.

II Hair

A *Differentiation of Cortical Cells*[3]

The cells of the *matrix* of an active hair follicle are round and small (Fig 1), and possess a high potential for mitotic activity. When the newly produced cells ascend to the level of the *pre-elongation* region of the hair bulb, they are still capable of dividing. Near the neck of the hair bulb, in the *elongation* region, the nuclei of these cells change from a round to an oval shape, minute fibrils develop in the cytoplasm, and the cells increase in size As the cells pass through the neck of the hair bulb and reach the *prekeratinization* region, they become still larger and become elongated, they reach their maximum size in the *keratogenous* zone. The cells in this zone contain thin, rod-shaped nuclei and a cytoplasm filled with long and coarse fibrils. The cytoplasmic fibrils are oriented with long axes parallel to the longitudinal axis of the hair In the precortex region, the cells coalesce, leaving empty spaces which at lower levels are filled with fluid, but in the *mature cortex* are filled with air

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[2] Because of the paucity of data for human hair, occasional reference will be made to similar data available for nonmedullated sheep wool fibers, structures considered generally comparable to human hair

[3] *Terminology is adapted from W Montagna (1956)*

B. Characteristics of Mature Cortex and Properties of Hair Keratin

The mature hair cortex appears as a transparent, homogeneous horny mass, and single cortical cells cannot be distinguished in it. The cortex forms about 90% of the wool fiber and of most hairs.

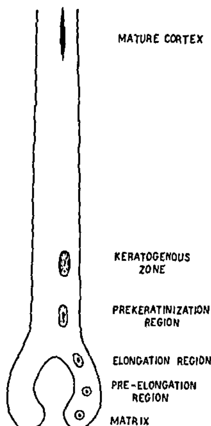


FIG 1 Schematic illustration of differentiation of cortical cells of the hair

The main component of hair and wool is water-insoluble. Keratin is present in the largest quantity, insoluble membranous substances such as the epicuticle, cell membranes, etc, form a relatively small part of these structures (Schuringa *et al*, 1952) Water-soluble substances such as pentose, phenols, uric acid, glycogen, glutamic acid, valine, and leucine have been identified in minute quantities (Bolliger, 1951).

Hair and wool keratin is built up of amino acids Cystine is present in large quantities, varying from 11 to 18% Histidine, lysine, and arginine occur in the characteristic molar proportion 1 4.12 (Block,

1939). The amino acids of wool, as listed in "Handbook of Biological Data" (Spector, 1956) are shown in Table I.

TABLE I
AMINO ACIDS OF WOOL KERATIN[a]

Amino acid	*Per cent*[b]
Glycine	6 8
Alanine	4 0
Valine	5 4
Leucine	8 6
Isoleucine	4 3
Phenylalanine	4 0
Proline	8 0
Serine	9 9
Threonine	6 5
Tyrosine	5.5
Aspartic acid	7 4
Glutamic acid	14 0
Arginine	10 6
Lysine	3 3
Histidine	1 1
Tryptophan	1.5
Cystine	13 6
Methionine	0 7

[a] From Spector (1956)
[b] Per 100 gm of protein on a moisture-free and an ash-free basis.

Hair and wool keratin contain amino acids in different sequences and groupings (Blackburn, 1950, Middlebrook, 1949, 1951, Speakman, 1952). On the basis of end-group analyses it is assumed that at least seven different types of polypeptide chains occur in it (Ward and Lundgren, 1954) The polypeptide chains are considered to be arranged in the α-helical configuration of Pauling and Corey (1951). The helices are stabilized by intramolecular hydrogen bonds between amide groups of one turn of the helix with the amide groups of the preceding and succeeding turns (Flory, 1956) Strong covalent bonds, such as the —S—S— bonds, occur mainly between cystine residues of polypeptide chains A few —S—S— bonds are also assumed to be along the main chains (Alexander, 1951, Alexander *et al*, 1950, Mercer and Olofsson, 1951b) Weak bonds, such as salt linkages and van der Wall's forces, also combine and hold together the polypeptide chains, adding to the strength of keratin structure (Alexander, 1951) This *intensely cross-linked system forms a highly resistant network of* practically unlimited molecular size and molecular weight. Several attempts were made to break down keratin in order to identify its com-

ponent molecular building units So far only extremely large units and very small ones have been identified. Electron microscope studies of wool cortex show rod-shaped particles measuring about 2000 A in length and 110 A in width (Farrant *et al.*, 1947). Molecular kinetic studies of wool, dispersed in 3.5 *N* sodium sulfide, indicate elongated molecules measuring about 170 A in length and 11 A in width, corresponding to the approximate size of a polypeptide chain (Olofsson and Gralén, 1950). By the use of saturated urea in bisulfite solution at 50°C, somewhat larger molecules were identified, measuring about 1150 A in length and 12.8 A in width (Mercer and Olofsson, 1951b).

According to this, keratin of the hair cortex can be considered to be laid down in the form of submicroscopic fibrils with parallel and cross-linked polypeptide chains This anisodimensional system shows characteristic physical properties X-ray diffraction studies reveal a crystalline structure of the α-keratin type (Astbury and Woods, 1934, Giroud and Champetier, 1936, Giroud and Leblond, 1951, MacArthur, 1943). The composite bodies give rise to positive double refraction with respect to the long axis of the system (Schmidt, 1924). The magnitude of double refraction in wool was measured as 0.011 to 0.013 (Mercer, 1949). If hair or wool is heated to about 130°C, it shrinks in the longitudinal direction In cold water, it can be stretched to about 50 to 70% of its original length (Frey-Wyssling, 1948) In a steam bath, it can be reversibly stretched to about twice its original length (Astbury and Woods, 1934)

Hair and wool keratin are insoluble in aqueous salt solutions, in weak acids, in weak alkalies, and in saturated neutral urea solution They also resist the action of trypsin. Human hair normally does not contain more than 9% water (Voit, 1930). If it is suspended in water, its water content may increase up to about 30% (Chamberlain and Speakman, 1931, Valko and Barnett, 1952). In acid solutions, between pH 1 and 2, moderate lateral swelling occurs In alkaline solutions at about pH 10, lateral swelling is intense At pH 12, unlimited swelling takes place and keratin dissociates, the components passing into solution.

Dissociation of keratin has been readily achieved by chemicals which open peptide bonds, reduce —S—S— bonds, or break hydrogen bonds. The most effective solubilization is obtained with sodium sulfide (Jones and Mecham, 1943a, Olofsson and Gralén, 1950), potassium cyanide (Gillespie and Lennox, 1953), alkaline thioglycolate (Gillespie and Lennox, 1955a, b), mercaptoethanol (Jones and Mecham, 1943b), chlorine dioxide (Das and Speakman, 1950), peracetic acid (Alexander *et al.*, 1950), and urea bisulfite solution at 50°C (Friend and O'Donnell, 1953, Mercer and Olofsson, 1951b, Ward, 1952, Woods, 1952). Goddard and

Michaelis (1934) found that native sheep wool can be completely solubilized within a few hours at 30°C in 0.5 *M* sodium sulfide or 0.5 *M* thioglycolate adjusted with alkali to pH 12, or in a mixture of 0.5 *M* sodium cyanide and 0.1 *N* sodium hydroxide. The soluble wool preparations contain practically the same amount of sulfur, nitrogen, and cystine as did the original material. The isoelectric point of soluble wool preparations is estimated to be between pH 4.1 and 4.7.

The action of solubilizing agents on keratin appears to be complex rather than specific. Dissociation of keratin appears to depend upon the manner and degree to which cohesive bonds are reduced and upon the number of chain molecules which are ruptured. Molecular kinetic studies reveal a considerable variety of products. The molecular weight of solubilized keratins ranges from 10,000 to about 80,000 (Friend and O'Donnell, 1953, Mercer and Olofsson, 1951b; Middlebrook, 1951, Olofsson and Gralén, 1950, Ward, 1952, Ward and Lundgren, 1954). Fragments of keratin, which show an average molecular weight of 70,000 and contain a few polypeptide chains held together by a few intact —S—S— bonds, are assumed to represent a basic building unit of native wool keratin.

C. *Characteristics of Differentiating Cortical Cells and Properties of Prekeratin*

In the *keratogenous* zone, the presumptive cortical cells appear as distinct hydrated units. The cells contain flattened nuclei and numerous coarse cytoplasmic fibrils (Fig. 1). Synthesis of nucleic acids is very low in this region, as indicated by studies with radiophosphorus (Bern *et al.*, 1955). Studies with radiocystine and radiomethionine show that sulfur-containing amino acids are taken up in large quantities and are incorporated into the protein constituents (Bélanger, 1956). The cytoplasm of the cells can be stained with both basic and acidic dyes (Montagna, 1956), whereas the fully cornified cells show affinity only for acidic dyes (Auber, 1950-1951). The cytoplasmic fibrils can be best seen in polarized light. They reveal intense double refraction which is of identical value to that of mature keratin (Mercer, 1949). X-ray diffraction studies show an α-type keratin structure. This prekeratin, however, is not fully stabilized. If a plucked hair is heated to 90°–95°C, the keratogenous zone collapses without contraction. The cells show moderate swelling in distilled water, and are dissociated in saturated urea when the hydrogen bonds of prekeratin are opened (Mercer, 1949). Histochemical studies show that —S—S— bonds are not yet formed. The prekeratin contains only free —SH groups in large quantities

(Barrnett and Seligman, 1953, 1954; Hardy, 1952; Montagna *et al*, 1954, Odland, 1953).

The cells of the *prekeratinization* and *elongation* regions (Fig 1) are metabolically more active than those of the keratogenous zone and contain nucleic acids in large quantities (Montagna, 1956). Radioautographic studies with P^{32} indicate an intense rate of synthesis of nucleic acids in these cells (Bern *et al.*, 1955). Radiocystine and radiomethionine are taken up in smaller quantities than in the keratogenous zone, indicating a lesser degree of incorporation of sulfur-containing amino acids into proteins (Bélanger, 1956, Bern *et al.*, 1955). In addition to small cytoplasmic fibrils, these cells also contain a considerable amount of solid material, and the cytoplasm shows affinity for basic dyes (Auber, 1950-1951) The fibrils reveal weak double refraction in polarized light (Mercer, 1949, Montagna, 1956). Under the electron microscope, fine filaments can be seen in the cytoplasm of these cells. The cell structure is quite labile, it swells in distilled water and rapidly dissociates in saturated urea (Hardy, 1952, Mercer, 1949) X-ray diffraction studies reveal no characteristic pattern in the elongation region An α-pattern is apparent only at the level of the prekeratinization region (Mercer, 1949) Histochemical preparations show a moderate amount of protein-bound free —SH groups in the cells of these regions (Hardy, 1952, Montagna *et al*, 1954, Odland, 1953)

Biochemical studies show that there is less sulfur in *wool fiber roots* than in mature wool fibers (Ellis *et al*, 1950). Sulfur was identified in wool roots in both the —SH and —S—S— forms. The presence of —S—S— bonds in the wool root is not fully understood. About 69% of total —SH sulfur of the wool root was found to be nonextractable. About 29% was found to be extractable but nondialyzable, and only 4% was found to be dialyzable It is thought that the major portion of —SH might already be present in the keratin precursor because most of the —SH sulfur is of the nonextractable type.

Glutamic acid, glycine, alanine, valine, leucine, isoleucine, histidine, lysine, arginine, serine, aspartic acid, taurine, and cysteic acid were identified at the site of formation of prekeratin in the wool root (Ellis *et al*, 1950) All of these, with the exception of taurine and cysteic acid, are found in the mature wool keratin

D The Mechanism of Keratin Formation in the Hair Cortex

The production of keratin precursors probably starts in the cells of the *upper bulb.* The initial phase of keratin formation in these cells is obscure It is not clear if the presumptive cortical cells synthetize a

specific keratin precursor with an amino acid composition characteristic for final keratin directly, or if the keratin precursor corresponds to a simple cytoplasmic protein which will gradually be converted into keratin by a specific mechanism.

A fibrous keratin precursor apparently first appears in the form of fine submicroscopic filaments. These increase in number, associate laterally, and eventually form microscopic fibrils which can first be seen in the cells of the *elongation* region. They reveal weak double refraction in polarized light, indicating some organization at the submicroscopic level. These fine fibrils have relatively few cohesive bonds and appear to be poorly stabilized.

The cytoplasmic fibrils in cells of the *prekeratinization* region appear to be aligned parallel to the longitudinal axis of the hair and correspond to the final orientation of the molecules of mature keratin. In the organization of prekeratin, the natural tendency of asymmetric particles to aggregate in parallel fashion might be a prevailing factor (Flory, 1956). Morphodynamic forces, however, might also play a role Huxley's layer forms a rigid keratinized funnel at the neck of the hair bulb. As the cells ascend and pass through this funnel, they may be under lateral pressure and become oriented by shear forces (Mercer, 1949, Montagna, 1956)

The higher amount of protein-bound —SH groups in the prekeratinization region might be due either to increased production of prekeratin or increased incorporation of sulfur-containing amino acids into the cytoplasmic fibrils The prekeratin of this region is still weakly stabilized, it swells in distilled water and dissociates in saturated urea

The coarse cytoplasmic fibrils in cells of the *keratogenous zone* incorporate a large amount of sulfur-containing amino acids and reveal a large quantity of —SH groups. The organization of keratin molecules at this level appears to be complete, since the fibrils reveal a structure characteristic of α-keratin and show double refraction identical to that of mature keratin, final organization apparently takes place at the submicroscopic level. This prekeratin is more firmly stabilized by hydrogen bonds.

The final stage of keratin formation consists of coalescence, consolidation, and hardening of cytoplasmic fibrils in the cells of the precortex and *mature cortex* High stability is achieved by the formation of a dense, dry structure and by the oxidation of all —SH groups into —S—S— bonds. In the oxidation of —SH groups, copper has been considered as a catalyst (Marston, 1946). The mechanism of —S—S— bond formation is not fully understood. The presence of a cementing

substance between the coalescing fibrils has been assumed and might constitute an important part in the formation of mature keratin The chemical nature of this cementing substance is not well known (Stoves, 1947, Ward and Lundgren, 1954).

III. Epidermis

A. Differentiation of Epidermal Cells

The *basal* cells of the epidermis are cuboidal, columnar, or fusiform, the *spinous* cells are polyhedral at lower levels and increasingly flattened at higher levels (Fig. 2). Both contain tonofibrillae oriented

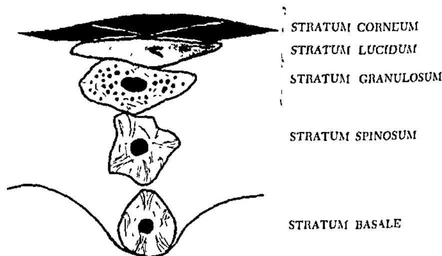


Fig 2 Schematic illustration of differentiation of epidermal cells

preferentially in a plane perpendicular to the skin surface (Matoltsy and Sinesi, 1957) As the spinous cells ascend and change to *granular cells,* they increase in size In the cytoplasm, numerous keratohyalin granules develop, tonofibrillae appear fragmented and mitochondria disappear The cells of the *stratum lucidum* are flattened The nuclei disintegrate and keratohyalin granules cannot be recognized as single bodies The cells of the *stratum corneum* are strongly flattened and tightly associated, mainly by toothlike edges at their periphery. In some cornified cells, remnants of nuclear membranes can still be recognized. The cytoplasmic components coalesce and form homogeneous appearing horny masses

B. *Characteristics of Stratum Corneum and Properties of Epidermal Keratin*

The stratum corneum of the epidermis is marked by irregular grooves. That of thick epidermis, as on the fingers or soles has regular deep grooves and high ridges (Fig. 3) Polarization optical studies of thick stratum corneum of human plantar skin (Matoltsy and Odland,

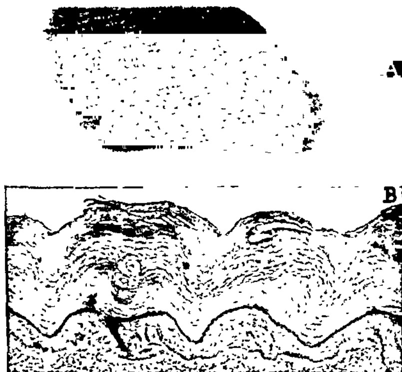


FIG 3 A Human plantar skin showing regularly arranged grooves and ridges on the surface B Cross section of human plantar skin showing a thick stratum corneum

1955, 1956) show that in groove areas keratin is oriented parallel to the skin surface in the direction of the grooves (Fig 4). In ridge areas, the structure is more complicated because the individual cornified cells are randomly oriented In the crossbands of ridge areas, keratin is oriented in a transverse direction, being at right angles to that of the groove areas The circumferentially oriented cells around the sweat ducts give rise to a continuous annular structure. In other parts of ridge areas, the cells are irregularly disposed and show a randomly oriented keratin structure



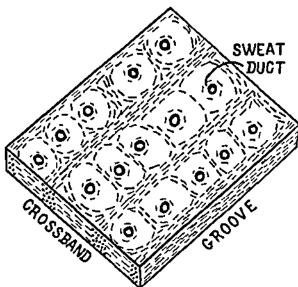


FIG 4 Upper picture shows an unstained horizontal section of thick stratum corneum of human plantar skin in polarized light Parallel birefringent lines correspond to groove areas, the birefringent crossbands are component structures of ridge areas *Lower picture shows schematically the orientation of keratin in various portions of thick stratum corneum*

The groove and ridge areas of thick stratum corneum stain differently (Patzelt, 1926) and they show differences in reactivity to alcohol and distilled water. Ridge areas shrink more in alcohol and swell more in distilled water than the groove areas do (Cauna, 1954).

X-ray diffraction studies reveal an α-keratin structure in the stratum corneum (Derksen and Heringa, 1936, Derksen *et al*, 1937, Giroud and Leblond, 1951, Rudall, 1947, 1952). Epidermal keratin shows a positive crystalline double refraction (Fig. 5) and no form double refraction (Matoltsy and Balsamo, 1955a). The value of crystalline double refraction was determined as 21.8×10^{-4}. The lack of form



FIG 5 A single extracted cornified cell of human plantar skin is shown in polarized light. It consists mainly of epidermal keratin

double refraction indicates that the keratin molecules are densely packed and are not separated by definite phase boundaries. Electron microscope *studies of the stratum corneum show a dense and non-fibrous* structure (Selby, 1956) Thermal contraction of stratum corneum is at about 80°C, or approximately 50°C less than the temperature at which thermal contraction occurs in the hair. In a steam bath, the stratum corneum can be stretched from 100 to 150% of its original length (Rudall, 1952).

Both histochemical and biochemical studies reveal the presence of —S—S— bonds as well as free —SH groups in the stratum corneum, indicating that epidermal keratin is not as fully stabilized by —S—S— bonds as is hair keratin (Eisen *et al*, 1953, Goldblum *et al*, 1954, Van Scott and Flesch, 1954a, b) *In the stabilization of epidermal keratin*, hydrogen bonds, salt linkages, and van der Wall's forces are considered to play a similar role as in hair keratin.

The amino acids of the total stratum corneum, removed from the palmar skin of human beings, were studied by paper chromatography. The average values of these analyses (Muting *et al*, 1955) are shown in Table II.

Chemically, the stratum corneum is more *complex* than hair or wool. That of human plantar skin consists of approximately 20% soluble remnants of nuclear and cytoplasmic components (10% dialyzable substances and 10% nondialyzable material), 65% insoluble epidermal keratin, and 5% resistant membrane material. About 7 to 9% of ethanol-extractable lipids are also present. Thick stratum corneum normally contains about 10 mg water per 100 mg dry material In an atmosphere of about 100% humidity its water content might increase to 70 mg per 100 mg dry material (Blank, 1952). The thin stratum corneum on the skin of the abdomen, back, or forearm contains 30 to 42% soluble remnants of nuclear and cytoplasmic substances, an amount greater than that found in the thick stratum corneum (Grunberg and Szakall, 1955, Szakall, 1955). Lipids also occur in a somewhat larger amount, being about 12%

TABLE II
AMINO ACIDS OF TOTAL STRATUM CORNEUM OF HUMAN PALMAR SKIN[a]

Amino acid	Per cent
Glycine	3 7
Alanine	6 9
Valine	6 7
Leucine	10 2
Isoleucine	2 1
Phenylalanine	7 1
Proline	6 6
Serine	8 3
Threonine	6 0
Tyrosine	3 7
Aspartic acid	4 7
Glutamic acid	13 2
Arginine	4 3
Lysine	7 9
Histidine	2 9
Tryptophan	2 5
Cystine	9 1
Methionine	2 0

[a] From Muting *et al* (1955)

The *components* of the stratum corneum were studied in most detail in the thick human plantar skin (Matoltsy, 1956a, Matoltsy and Balsamo, 1955a, b). Isolated pieces of the thick stratum corneum (Fig 3A)

were ground in an Abbe pebble ball mill, using porcelain balls, at a constant speed of 115 rpm for 17 hours. The resulting powder consisted mainly of single cornified cells, a few cell clumps and some cell fragments (Fig 6). These isolated cornified cells can be fractionated into their constituent elements according to the scheme shown in Table III.

TABLE III

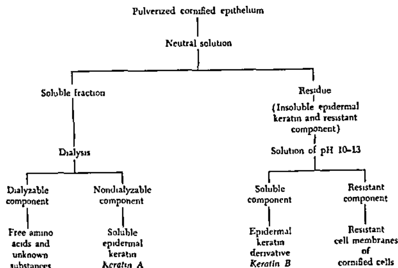




FIG 6 Photomicrograph of pulverized stratum corneum showing single cornified cells, cell clumps, and cell fragments

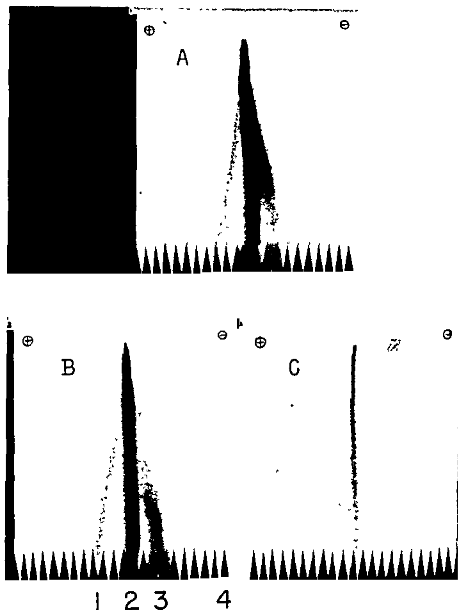


Fig 7 A Separating components of soluble fraction of pulverized stratum corneum of human plantar skin B Dialyzable component of the soluble fraction Line 1 is formed by acidic amino acids, line 2 by neutral amino acids and small peptides, lines 3 and 4 by basic amino acids C Nondialyzable component of the soluble fraction It consists of soluble epidermal keratin (Keratin A) Continuous-zone electrophoretic patterns were obtained after 3 hours of electrophoresis in Sørensen's phosphate buffer of pH 5 2 and developed by spraying ninhydrin solution on dry filter paper

The *soluble fraction* of pulverized cornified epithelium is best extracted with Sørensen's phosphate buffer at pH 7.1. Continuous-zone electrophoresis of this fraction reveals a very complex nature (Fig 7A). Only a portion of the soluble fraction passes through a collodion bag and approximately 60% is nondialyzable, the *dialyzable* component contains mainly small peptides and free amino acids (Figs. 7B and 8). The

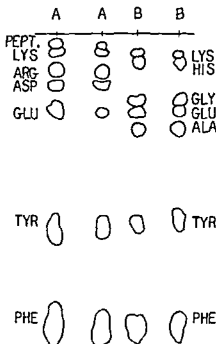


FIG 8 Tracings of a chromatogram of the dialyzable component of pulverized cornified epithelium of human plantar skin (AA) Tracings of a known amino acid mixture (BB).

nondialyzable component consists of a single protein (Fig. 7C) which was called Keratin A (Matoltsy and Balsamo, 1955a) and is considered to be a naturally occurring soluble type of epidermal keratin

Keratin A can be precipitated by *acidification* of the soluble fraction to about pH 4 and the precipitate can be redissolved at pH 7 1, and reprecipitated in the same manner Keratin A is electrophoretically homogeneous and has its isoelectric point at pH 4 1 (Fig 9) Keratin A contains 15 7% total nitrogen, about 0 3% cystine, and 1 7% total carbohydrates Tests for phosphorus, ribose, and deoxyribose nucleic acids are negative In the ultracentrifuge, Keratin A appears heteroge-

neous (Matoltsy, 1956a). About 40% sediments rapidly and shows a sedimentation constant of 1.7. About 42% of it sediments slowly and has a sedimentation constant of 1.2 (Fig. 10) At the present time, it is not known whether Keratin A corresponds to a precursor molecular

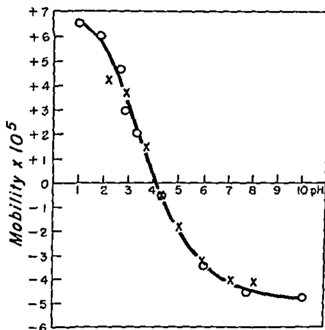


FIG 9 Mobility of Keratin A (x) and Keratin B (o) determined as a function of pH by Tiselius moving-boundary electrophoresis The isoelectric point of both Keratin A and Keratin B is at pH 4 1

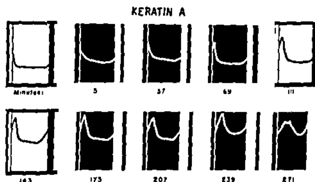


FIG 10 Tracings of schlieren diagrams of Keratin A. Solvent: 0 1 M glycine buffer of pH 8 9 Keratin A concentration 1 5%

species of epidermal keratin, or if it represents a natural degradation product of decomposing cornified cells

The *residue* (Table III) of powdered cornified epithelium consists of approximately 92% insoluble epidermal keratin and 8% resistant membrane material These two components cannot be separated without serious damage of their constituents. The amino acid composition of epidermal keratin, therefore, had to be determined by analyzing the *whole residue* *Columnar chromatographic analyses reveal the amino* acid composition shown in Table IV.

TABLE IV

AMINO ACIDS OF EPIDERMAL KERATIN ISOLATED FROM HUMAN PLANTAR SKIN[a]

Amino acid	Per cent[a]
Glycine	9 0
Alanine	—
Valine	2 3
Leucine	7.2
Isoleucine	2 6
Phenylalanine	—
Proline	—
Serine	6 9
Threonine	2.1
Tyrosine	—
Aspartic acid	3 9
Glutamic acid	14 2
Arginine	7 6
Lysine	6 9
Histidine	1 6
Tryptophan	—
Cystine	—
Methionine	0 9

[a] Per 100 gm of protein on a moisture-free and ash-free basis

This epidermal keratin preparation is stable in weak acids and in weak alkalies (Fig. 11) In acid solutions between pH 1 and 4, or in alkaline solutions between pH 8 and 10 moderate swelling occurs In alkaline solutions at about pH 11 9 unlimited swelling takes place leading to dissociation of epidermal keratin, this can also be achieved by reducing substances and mixtures of reducing substances with alkali and alkaline urea The solubilizing effect of such chemicals is demonstrated in Fig 12 Efficient solubilization of epidermal keratin can be achieved by using any of the following solvents (pH between 10 and 13). 0.02 *N* NaOH, 0 02 *M* Na_2S, 10 0 mM KCN in 0 02 *N* NaOH, 2 5 mM $CH_2SHCOOH$ in 0 02 *N* NaOH, and 50 0% $(NH_2)_2CO$ in 0 0? *N*

NaOH. Epidermal keratin, solubilized by any of the above solutions, was called Keratin B (Matoltsy and Balsamo, 1955a).

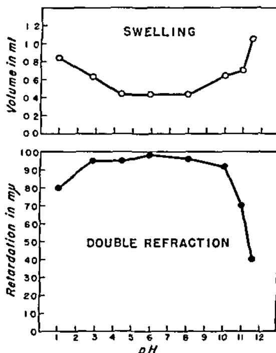


FIG 11 Upper curve shows volume of 100 mg pulverized cornified epithelium of human plantar skin in glycine-HCl and glycine-NaOH standard mixtures of different pH Lower curve shows values of double refraction (retardation) of a single cornified cell immersed in the same solutions In solutions, more alkaline than pH 11, *retardation is irreversibly decreased and abolished, implying dissociation* of epidermal keratin molecules

Keratin B can be precipitated at pH 4 and redissolved in mildly alkaline buffers. Characteristic chemical and physical data of Keratin B preparations are shown in Table V Keratin B, prepared by 0 02 *N*

TABLE V
CHEMICAL AND PHYSICOCHEMICAL DATA OF KERATIN B

Preparation obtained by	Per cent N	Per cent Carbohydrates	Per cent Cystine	Mobility $\times 10^5$
0 02 *N* NaOH	15 88	0 91	0 23	7 85
0 02 *M* Na_2S	15 57	0 24	0 23	8 29
10 0 mM KCN in 0 02 *N* NaOH	15 72	0 68	—	9 84
2 5 mM $CH_2SHCOOH$ in 0 02 *N* NaOH	15 68	0 57	—	8 41
50% $(NH_2)_2CO$ in 0 02 *N* NaOH	15 08	0 96	0 17	7 10

NaOH, is electrophoretically homogeneous. Its isoelectric point was determined at pH 4 1 (Fig. 9), coinciding with that of Keratin A In the ultracentrifuge about 35% of Keratin B sediments rapidly and shows a sedimentation constant of 3 8. About 56% sediments slowly and shows a sedimentation constant of 2.2 (Fig 13) (Matoltsy, 1956a). Keratin B preparations obtained by sodium sulfide, potassium cyanide, alkaline thioglycolate, and alkaline urea all reveal. (a) one large component,

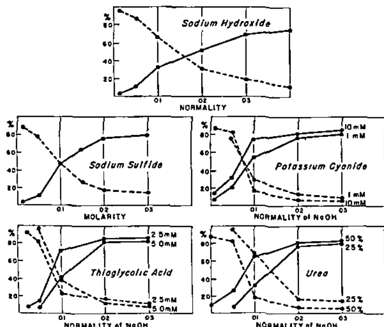


FIG 12. Percentages of nondialyzable (solid lines) and of undissolved fractions (broken lines) are shown after treatment of pulverized cornified epithelium of human plantar skin for 24 hours with alkali, reducing substance, and mixtures of reducing substances with alkali and alkaline urea.

(b) two small rapidly sedimenting components, and (c) one small, slowly sedimenting component. These results indicate that different solubilizing agents split epidermal keratin into particles of different molecular weights A homogeneous preparation, adequate for specific molecular weight determination, has not yet been obtained

Resistant membranes (Table III) can be isolated by suspending single cornified cells in 0 1 *N* NaOH The cells swell greatly in this solution, the cell membranes becoming ruptured, and the cell contents flowing out Most of the cell membranes which are collected by centrifuga-

tion appear wrinkled when viewed under the microscope (Fig 14A), but some retain the form of the original cell, indicating a fairly rigid structure (Fig. 14B). These membranes are resistant to strong reducing agents and do not dissolve in solutions like 0.5 *N* thioglycolate in 1 5 *N* sodium hydroxide, even after immersion for several days (Matoltsy and Balsamo, 1955a, b). One per cent sodium sulfide solution at 50°C was also used to dissolve the epidermis, collected from human sunburned backs, and again resistant cell membranes were isolated (Lagermalm *et al.*, 1951). These cell membranes examined under the electron microscope, were found to have a thickness of 100 A The cell membranes consist mainly of protein Columnar chromatographic analyses show the amino acid composition[4] to be as in Table VI

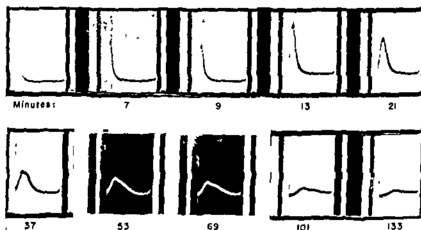


FIG 13. Photograph of schlieren diagrams of Keratin B Solvent 0 1 *M* glycine buffer of pH 8 9 Keratin B concentration 1 0%

C Characteristics of Differentiating Epidermal Cells and Properties of Prekeratin

The cells of the stratum lucidum (Fig 2) are flat but more hydrated and less hardened than the cells of the stratum corneum. They show positive double refraction in a plane parallel to the skin surface, indicating that prekeratin is oriented in a manner similar to that of the mature keratin (Fig. 15). Electron microscope studies reveal the hetero-

[4] Since the analyzed material was isolated by the use of 0 1 *N* sodium hydroxide which is known to damage various amino acids, the values presented in Table VI can be considered only approximate values

geneous structure of this layer having alternating phases of high and low densities. In areas of high density, hyalinized tonofilaments appear (Selby, 1956). Histochemical studies reveal a high concentration of protein-bound —SH groups (Barnett and Seligman, 1954; Eisen *et al*,

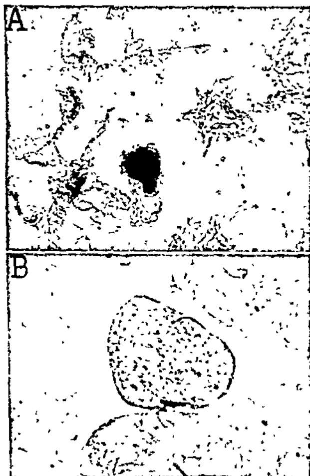


FIG 14 Isolated membranes of cornified cells of human plantar skin A Wrinkled membranes, collected by centrifugation B Empty cell envelopes resembling the original form of cornified cells

1953, Montagna *et al*, 1954) Studies with radiocystine and radiomethionine show that sulfur-containing amino acids are incorporated in these cells in large quantities (Bélanger, 1956; Bern *et al.*, 1955) If the stratum lucidum is exposed to water, it shows moderate swelling

Saturated urea penetrates this layer rapidly and causes dissociation of its cells (*Fig.* 16) (Matoltsy and Herbst, 1956b).

TABLE VI
AMINO ACIDS OF ISOLATED CELL MEMBRANES OF CORNIFIED CELLS OF HUMAN PLANTAR SKIN

Amino acid	*Per cent*
Glycine	5 6
Alanine	—
Valine	1 8
Leucine	5 5
Isoleucine	4 7
Phenylalanine	—
Proline	—
Serine	4 7
Threonine	2 5
Tyrosine	—
Aspartic acid	4 4
Glutamic acid	8 4
Arginine	1 5
Lysine	3 0
Histidine	1 2
Tryptophan	—
Cystine	—
Methionine	2 3

The *granular* cells are nucleated cells (Fig. 2) They reveal the presence of birefringent material only at the cell periphery. In the electron microscope, densely packed fine filaments with a hyaline-type structure can be seen distributed throughout the cytoplasm (Selby, 1956) The cytoplasm of granular cells is mildly reactive to tests for —SH groups Keratohyalin granules occur in both microscopic and submicroscopic dimensions in large quantities. They show a negative test for —SH groups. Most investigators consider them to be composed of protein and of some nucleic acids (Leuchtenberg and Lund, 1951). According to some views, keratohyalin represents a prekeratin substance (Favre, 1950) or cytoplasmic debris which is not involved in keratin formation (Flesch, 1956). It has also been proposed that the keratohyalin granules represent complex coacervates which are formed by removal of water from the solvation layers of interacting colloids (Matoltsy, 1954). Since the granular cells are in an advanced stage of differentiation, it is possible that some basic proteins (histone) are already released from the nucleus and interact with acidic proteins of the unstabilized cytoplasm (Matoltsy, 1956b). This may give rise to such intracellular entities as the keratohyalin granules. The observa-

tion of Mercer (Chapter 5, this volume) on the nature of keratohyalin granules sheds some light on their relation to keratin formation.

The *basal* and *spinous* cells (Fig 2) are rich in nucleic acids (Dempsey *et al*, 1950). Radiocystine and radiomethionine are incorporated in a small amount in these cells, indicating a low metabolism of sulfur-containing amino acids. In the electron microscope the birefringent tonofibrils are seen to be composed of numerous fine tonofilaments, measuring from 50 to 100 A in diameter (Selby, 1955). The tonofibrils,

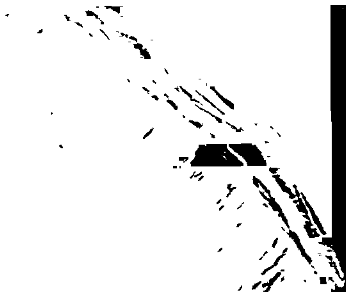


FIG 15 Unstained cross section of human abdominal skin in polarized light Most intense double refraction occurs in the stratum corneum and stratum lucidum Below this, birefringent material is oriented at right angles to the surface plane

which contain moderate amount of —SH groups, dissociate readily when exposed to the action of saturated urea (Fig 16) or of reducing substances (Carruthers *et al*, 1955a, Matoltsy and Herbst, 1956b). X-ray diffraction studies of the Malpighian layer reveal an α-keratin structure, possibly present in the tonofibrils. Alpha-keratin is weakly stabilized and shows thermal contraction at about 65°C, approximately 20°C less than the temperature at which thermal contraction is shown by epidermal keratin (Rudall, 1952).

A fibrous protein, called *epidermin* (Rudall, 1952) was isolated from the Malpighian layer of the parakeratotic epidermis of the cow's nose by the use of 6 *M* urea Films of dry epidermin preparations show

Saturated urea penetrates this layer rapidly and causes dissociation of its cells (Fig. 16) (Matoltsy and Herbst, 1956b).

TABLE VI

AMINO ACIDS OF ISOLATED CELL MEMBRANES OF CORNIFIED CELLS OF HUMAN PLANTAR SKIN

Amino acid	Per cent
Glycine	5.6
Alanine	—
Valine	1.8
Leucine	5.5
Isoleucine	4.7
Phenylalanine	—
Proline	—
Serine	4.7
Threonine	2.5
Tyrosine	—
Aspartic acid	4.4
Glutamic acid	8.4
Arginine	1.5
Lysine	3.0
Histidine	1.2
Tryptophan	—
Cystine	—
Methionine	2.3

The *granular* cells are nucleated cells (Fig. 2). They reveal the presence of birefringent material only at the cell periphery. In the electron microscope, densely packed fine filaments with a hyaline-type structure can be seen distributed throughout the cytoplasm (Selby, 1956). The cytoplasm of granular cells is mildly reactive to tests for —SH groups. Keratohyalin granules occur in both microscopic and submicroscopic dimensions in large quantities. They show a negative test for —SH groups. Most investigators consider them to be composed of protein and of some nucleic acids (Leuchtenberg and Lund, 1951). According to some views, keratohyalin represents a prekeratin substance (Favre, 1950) or cytoplasmic debris which is not involved in keratin formation (Flesch, 1956). It has also been proposed that the keratohyalin granules represent complex coacervates which are formed by removal of water from the solvation layers of interacting colloids (Matoltsy, 1954). Since the granular cells are in an advanced stage of differentiation, it is possible that some basic proteins (histone) are already released from the nucleus and interact with acidic proteins of the unstabilized cytoplasm (Matoltsy, 1956b). This may give rise to such intracellular entities as the keratohyalin granules. The observa-

urea from the human epidermis (Matoltsy and Herbst, 1956b, Roe, 1956a, b). In the epidermis of human skin, large quantities of *soluble proteins* are extractable with neutral or slightly alkaline buffers (Fig 17) (Matoltsy and Herbst, 1956a; Spier *et al*, 1954). These proteins supposedly are present in the Malpighian layer. They have not yet been

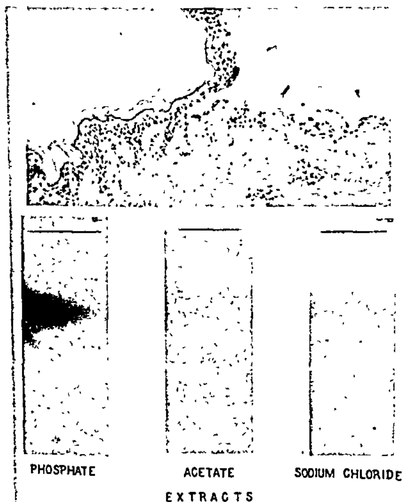


FIG. 17. Cross section of human abdominal skin showing separation of epidermis from dermis after incubation at 58°C Extracts of separated epidermis were obtained by 1/15 *M* phosphate buffer of pH 7 2, veronal-acetate buffer of pH 8 3, and a mixture of 1 0 *M* sodium chloride in 1 15 *M* phosphate buffer of pH 7.2 Electrophoresis pattern of each extract (lower picture) reveals two distinct components

sufficiently characterized to enable one to draw conclusions about whether they originate from tonofibrils or from other cell constituents

In a biochemical study Van Scott and Flesch (1954a, b) report equal concentrations of —S—S— bonds in the cells of the Malpighian layer and in the cornified cells of human epidermis and postulate that keratin, containing —S—S— bonds, is already formed at the base of the epidermis This viewpoint awaits coordination with histochemical and radioautographic findings. Radioautographic studies indicate the highest uptake of sulfur-containing amino acids to be below the stratum corneum and histochemical studies show abundant —S—S— bonds to be formed only in the stratum corneum

D *The Mechanism of Keratin Formation in the Epidermis*

The mechanism of keratin formation in the epidermis is still a problem. A gradual increase in number and thickness of fibrous elements in the cytoplasm of differentiating epidermal cells is not as apparent as it is in the differentiating cortical cells of the hair. Furthermore, the amount of tonofibrillae produced by the differentiating epidermal cells, does not seem to account for the amount of keratin occurring in the cornified cells.

In the granular cells, in addition to fibrous elements, keratohyalin granules and nonfibrous substances are also present in relatively large quantities. Which component of granular cells represents a prekeratin substance and which one will be eliminated or utilized in the formation of final keratin is not clear at the present time Rothman (1954) proposed the idea that the cell components might first dissociate and then reunite in order to form a final keratin. This is a probable mechanism for the formation of final keratin in the epidermis.

Below the stratum corneum, at the level of the keratinous zone, a *prekeratin is formed with a relatively high sulfur content* Flattening of cells and loss of their water content might create favorable conditions for the prekeratin molecules to aggregate and unite into a highly organized structure. The oriented prekeratin molecules appear weakly stabilized and are held together only by electrovalent and hydrogen bonds. Thus prekeratin contains no strong covalent bonds, such as the —S—S— bonds, only —SH groups are present in large quantities, as *indicated by histochemical studies.*

The final step in the formation of epidermal keratin consists of intense withdrawal of water and association of molecules into a densely packed structure. The —SH groups of prekeratin become oxidized only partially to —S—S— bonds. This fact accounts for the relatively poor stability of epidermal keratin

The *potential* of epidermal cells at various layers when considered from the viewpoint of keratinization appears to be quite different. Adequate systems for keratin synthesis and for stabilization of keratin seem to develop and work efficiently only above the basal layer. This was observed in tissue cultures of adult human abdominal skin (Matoltsy and Sinesi, 1957). When differentiation was induced by the removal of the stratum corneum and proliferation was inhibited by adequate culturing techniques, both spinous and granular cells changed *in situ* into keratin-containing anucleated horny cells The basal cells did not change but maintained their undifferentiated state (Fig 18).

IV. Discussion

The *initial* mechanism of keratinization in either the hair cortex or in the epidermis is not clear For a better understanding of the initial phase of keratinization, more knowledge is necessary on properties of the differentiating epithelial cells and on the nature of the keratin precursor substances.

In the *early* stage of keratinization, cytoplasmic fibrillation appears to be a basic mechanism which *later* becomes associated with the decomposition and elimination of certain cytoplasmic and nuclear elements Although these are common properties of keratinizing cells of both hair cortex and epidermis, the actual mechanism of keratinization is different. In the differentiating cells of the hair cortex the cytoplasmic fibrils gradually reach a high concentration and the cells practically consist of fibrils when the nuclear and cytoplasmic activities cease and an efficient elimination of nonkeratin constituents takes place. In the differentiating cells of the epidermis a gradual cytoplasmic fibrillation is less apparent and the elimination of nuclear and cytoplasmic nonkeratin components is also less effective than in the differentiating cells of the hair cortex

The *final* phase of keratinization takes place in "nonviable" cells In the cortical cells of the hair the cytoplasmic fibrils simply unite and form a hardened mature keratin Prekeratin of the epidermis appears to undergo complicated colloid-chemical and physicochemical changes as it forms a highly organized final mature keratin.

Differences can be recognized in the *chemical composition* of the keratin which is formed in both hair and in the epidermis. If Table I is compared with Table II or IV, it can be seen that the amino acid composition of wool keratin is basically different from that of epidermal keratin. This is consistent with the concept that there are different kinds of keratins, as implied by others by the terms "eukeratin" and "pseudokeratin." It would appear that either the availability of amino acids

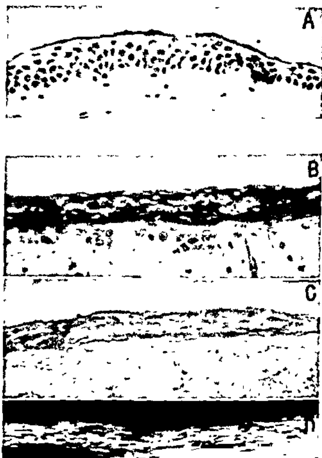


FIG 18 A Cross section of adult human abdominal skin from which the stratum corneum was removed by 30 strippings of the skin surface with Scotch Tape B, C and D Cross sections of stripped skin which was cultured in hanging-drop preparations for 7 days The preparation shown in B is stained with hematoxylin and eosin *Note the wide keratinized upper portion In preparation C* the —S—S— bonds were reduced to —SH before application of *Barnett and* Seligman's technique Note intensely reactive upper portion of the epidermis Preparation D is unstained and was photographed in polarized light Most intense double refraction occurs at about the middle of the epidermis, an area corresponding to newly formed keratogenous zone (From original of Plate 1A Figs 2 and 4, Plate 1B Fig 4 and Plate 2 —S—S— Fig 4, Matoltsy and Sinesi (1957), p 65)

might be different in the hair follicles and the epidermis, or that keratin synthesis proceeds according to different principles in these keratinizing tissues.

Epidermal keratin is not only of different composition but it is also less resistant to physical and chemical agents than hair or wool keratin. The *relative weakness* of epidermal keratin is, however, compensated by the presence of cell membranes which consist of material of considerable rigidity and high resistance These cell membranes appear to be specific protective elements of single cornified cells, and together with epidermal keratin form a most efficient protective system along the entire surface of the organism.

V. Acknowledgments

Some illustrations in this paper have already appeared elsewhere. The author wishes to thank the representative editors for permission to reproduce Figs. 15 and 18 from the *Anatomical Record*, Figs. 3 to 14 and Tables III and V, from the *Journal of Biophysical and Biochemical Cytology*, and Figs. 4, 9, 16, and 17 from the *Journal of Investigative Dermatology.*

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CHAPTER 8

The Mitotic Activity of the Follicle

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I. INTRODUCTION

Although the bulb of a hair follicle is an epidermal derivative, its pattern of mitotic activity is strikingly different from that of the surface epidermis While the rate of cell replacement in the surface epidermis is relatively steady, varying only within narrow limits according to diurnal and hormonal rhythms (Bullough, 1952, 1955b), the rate of cell production in the follicle is rhythmic, varying sharply between the violent mitotic activity associated with the formation of a new hair and the total lack of activity once that hair has been formed. There may therefore be physiological differences between the mitotic activity of the surface epidermis and that of the growing follicle

A physiological study of the mitotic activity of the normal epidermis of the mouse has already been undertaken and the results have been reviewed by Bullough (1952, 1955b). In brief, the following conclusions can be drawn.

1. Active mitosis only occurs when the epidermal cells are able to absorb adequate supplies of carbohydrate and of oxygen. These are normally obtained from the blood capillaries, the carbohydrate being in the form of glucose Some oxygen may enter through the skin surface and, as in the cornea, some carbohydrate may be in the form of lactic acid derived from the dermis In either case the carbohydrate is oxidized through the Krebs cycle to provide the energy necessary for mitosis

2. This energy is evidently stored in some readily available form during the antephase with the result that, once a division has visibly commenced, the cell does not need to produce any further significant amounts of energy. Cell division is thus an all-or-none reaction, and even the death of the animal does not arrest it (Bullough, 1950b).

3. In a normal animal the maximum epidermal mitotic activity is seen during rest and sleep, and the minimum during active muscular exercise While the precise reason for this has yet to be demonstrated, it is possible that it is basically due to the opening of the surface capillaries of the dermis during sleep, and to their closure during muscular activity. Probably the mitotic inhibition seen in an active animal is due to a shortage of glucose and oxygen in the epidermis. When inhibited skin is immersed in a saline culture medium containing glucose and with a gas phase of oxygen, mitotic activity commences at once.

4 A further factor limiting mitotic activity in the epidermis seems to be the relative inefficiency of the glucokinase reaction whereby glucose is enabled to enter the cells. When this reaction is stimulated, for instance, by either insulin or an estrogen, the result is an increased epidermal mitotic rate

5 The general conclusion may be drawn that cell replacement in the epidermis is dependent on active respiration within the basal cells, although mere epidermal survival can be ensured by glycolysis alone (Medawar, 1947) Any factor which stimulates respiration also stimulates cell division, while any factor which inhibits either respiration or the processes of energy transfer also inhibits cell division.

With these conclusions in mind, the following study of the mitotic activity of the hair follicle was carried out using Strong's CBA and C^{57} mice In 2-month-old animals a new hair coat is produced by a wave of follicular activity which starts on the belly; passes up the sides of the body, and ends dorsally. After the body hair had been removed with electric clippers, the region of new hair growth could easily be determined, and the subsequent studies were confined to this region

For the study of follicular activity *in vivo* it was standard practice for 0.1 mg colcemid in 0.25 ml saline to be injected subcutaneously 5 hours before killing For the *in vitro* study the animals received no colcemid After they were killed, their skin was removed, and, by means of a high-speed rotary punch, small circles (approximate diameter 5 mm) containing active follicles were cut out These skin samples were immersed in a phosphate-buffered saline medium Details of this medium and of the *in vitro* technique are given by Bullough and Johnson (1951a)

Briefly, this technique depended on the immersion of up to 5 skin

samples, each from a different mouse, in 4 ml saline in a Warburg flask maintained at 38°C and constantly shaken. The mitoses present in the skin when it was separated from the body were allowed to go to completion during the first hour, and then 0.04 ml saline containing 0.016 mg colcemid were added from the side arm to arrest all the newly-developing mitoses in the metaphase. After 4 further hours the experiment was ended.

The skin samples were fixed in aqueous Bouin, cut at 7 μ to give longitudinal sections of the follicles, and stained in Ehrlich's hematoxylin and eosin to show the mitoses. To demonstrate the distribution of glycogen, other sections were fixed in Zenker-formol, and stained by the periodic acid-Schiff method.

In estimating the numbers of mitoses present, the cell matrix of the hair root was regarded as a homogeneous tissue. The follicles were examined in strict sequence along the length of the skin sections, and the total number of mitoses arrested by colcemid were counted in each follicular matrix. All follicles were included in the count, whether the section passed centrally through the matrix or whether it included only a small slice of the outer matrix edge. From each animal an average was derived from 50 such counts, and since each experimental group normally consisted of skin taken from between 5 and 10 mice, there were between 5 and 10 figures from which a grand average and standard error were derived.

II. Growth Cycle of the Follicle

A. *The Resting Hair Follicle (Telogen)*

In such a follicle two physiologically distinct regions can be discerned easily, the one above the level of the sebaceous gland duct and the other below. In the upper region, the cells of which are in direct continuity with the surface epidermis, the mitotic activity of the basal cell layer is similar to that of the basal layer of the epidermis, but in the lower region the cells are mitotically inert. In the upper follicle there is an open channel between the follicle wall and the hair shaft. In the lower follicle the walls press closely on to the hair shaft which, at its base, has a strong brushlike attachment to the surrounding cell mass. Beneath the follicle base is the small dermal papilla.

B. *Early Follicle Growth (Anagen I)*

The first sign of that mitotic activity which results in the production of a new hair is seen in the basal cells of the lower follicle, which then grow downwards as a solid column of undifferentiated and dividing cells to surround the dermal papilla. There is no mitotic activity in that part

of the lower follicle which surrounds the brushlike base of the old hair, while in the upper follicle normal mitotic activity continues (Fig. 1).

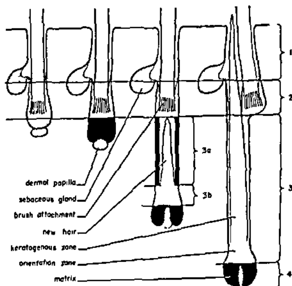


FIG 1 Diagrams of elongating hair follicles to show the areas of high mitotic activity (shaded black) Notice the four separate regions: (1) The upper follicle; (2) Zone of no mitosis, (3) Zone in which mitoses are transiently present during follicle elongation, and (4) The matrix

C. *Later Follicle Growth (Anagen II and III)*

As the new follicle elongates rapidly, the inner follicle sheath and the tip of the newly forming hair begin to differentiate, and the cells involved cease to show mitosis. From the base upward the sequence of zones is as follows:

(a) The basal region of very high mitotic activity takes the form of a ring which surrounds the dermal papilla. All the cells for the new hair shaft are formed in this region.

(b) A narrow region of differentiation in which no mitoses are seen

(c) A rapidly elongating region contains centrally the newly-differentiating hair and peripherally a zone of active mitosis in which are produced many of the new cells involved in the lengthening of the follicle Until the follicle is fully grown the lengthening of the follicle keeps exact pace with the lengthening of the new hair shaft Consequently, the hair tip remains static beneath the brushlike attachment of the old hair

(d) The nonmitotic zone surrounding the brushlike attachment of the old hair remains unchanged

(e) The cells of the upper follicle now show an increased rate of mitosis, and a similar increase in the mitotic activity takes place in the overlying surface epidermis (Chase *et al*, 1953).

D. *The Fully Grown Follicle (Anagen IV to VI)*

When its growth is complete, the follicle is at least six times longer than it was in the resting condition From the base upward the sequence of zones is as follows:

(a) The cells of the basal ringlike cell matrix show violent mitotic activity which, however, ceases abruptly at a point level with the tip of the dermal papilla.

(b) Above the dermal papilla is a zone in which the cells arrange themselves into columns and in which they begin to elongate and differentiate No mitotic activity is present.

(c) Above this is the keratogenous zone in which the cells are keratinized to form a recognizable hair. No mitotic activity is seen in any of these cells or in the cells of the surrounding follicle wall.

(d) With the point of the new hair now forcing upward, the brushlike attachment of the old hair and the surrounding cells are pushed to one side These surrounding cells show no mitotic activity

(e) The cells of the upper follicle show a subnormal mitotic rate, and a similar subnormal mitotic rate is found in the overlying epidermis (Mottram, 1945)

E. *The Final Stage (Catagen)*

When the new hair is fully grown, mitotic activity in the basal ringlike cell matrix suddenly ceases, and the new hair develops a brushlike attachment to the cell mass which surrounds its base. There follows the rapid degeneration and destruction of a greater part of the lower follicle. This is a complex and orderly procedure by which the follicle returns to its normal resting length.

III. Mitotic Activity of the Matrix

In a fully grown and fully active follicle the only mitotic activity is that of the ringlike matrix which surrounds the dermal papilla. A columnar epithelium of apparently static cells is based on the dermal papilla. Every division in these matrix cells is oriented in such a way that the new cell wall lies parallel to the surface of the dermal papilla, and the new cell is therefore pushed outward. In the first division of the new cell, which evidently takes place within a few hours, the position of the chromosome plate indicates that the long axis of the cell has tilted

upward through some 30° to 60°. The outermost of the two cells resulting from this division lies against the outer matrix wall, and on further division it can be seen to have turned through 90° so that its long axis points upward toward the newly forming hair. Thus, the new cells leaving the basal epithelium on the dermal papilla swing outward and upward, dividing as they go, to provide a steady stream of cells up the center of the follicle (Fig 2).

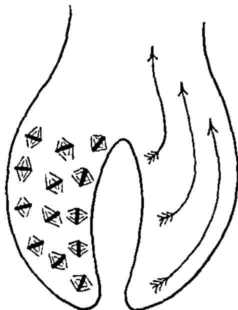


FIG 2 The orientation of mitotic figures in the matrix A new cell originates from the columnar epithelium which ensheaths the dermal papilla and its axis turns through 90° as it travels outward and upward.

The mitotic activity in the whole matrix is violent. By the use of colcemid it can be seen that, in a period of 4 hours, some 30% of the matrix cells entering division are arrested in metaphase. Making the assumption that the colcemid itself is without effect on the number of cells entering division, this would indicate that about 7.5% of the matrix cells enter mitosis during each hour On the further assumption that all the matrix cells are equipotent, this would also indicate that every cell undergoes division once in approximately 13 hours. This exceptionally high rate is in extreme contrast to the situation in the surface epidermis (Bullough, 1950a).

The results of the experiments performed to discover the approximate duration of each mitosis are given in Table I. Some 18 mitoses develop

in 4 hours in the amount of matrix represented by an average random section (7 μ thick). After the fourth hour, the colcemid treatment results in a mitotic inhibition so that there are still only 18 mitoses present after 5 hours From this information the following rough calculation may be made. Approximately 4 5 mitoses reach the metaphase per hour, but at any one moment (in the absence of colcemid) only about 2 mitoses can be seen at some stage between prophase and telophase Thus each mitosis must normally be completed in between 15 and 30 minutes This conclusion, although only approximate, is in marked contrast to the situation in surface epidermis, where the duration of a mitosis may be four or five times as long (Bullough, 1950a). Evidently, therefore the follicular matrix is unusually active not only in terms of the high proportion of cells undergoing mitosis per day, but also in the extreme speed with which these mitoses are completed

TABLE I
EFFECTS OF COLCHICINE ON MITOSIS[a]

No colchicine	Time after injection 0 1 mg colcemid per mouse				
	1 hr	2 hr	3 hr	4 hr	5 hr
1 75 ± 0.11	2 77 ± 0 40	5 80 ± 0 41	13 04 ± 1.22	18.46 ± 1 13	18 22 ± 0 19

[a] Average numbers of mitoses in one hair bulb section 7 μ thick Numbers of mice studied per group = 4

IV Mitotic Activity and Respiration *in Vitro*

In view of the known dependence of epidermal mitosis on the active respiration of the epidermal cells (Bullough, 1952), a series of experiments was carried out to discover whether the mitotic activity of the follicular matrix is similarly dependent In the first experiment, performed *in vitro*, the importance of an oxygen gas phase and of a carbohydrate substrate was assessed (Table II) In this first experi-

TABLE II
EFFECTS OF GLUCOSE AND OXYGEN ON MITOSIS[a]

Gas phase	Substrate: Saline alone	Substrate: Saline + 0 02 M glucose
Nitrogen	0 (Slight necrosis)	0 (Slight necrosis)
Air	1 18 ± 0 15	2 13 ± 0 17
Oxygen	1 89 ± 0 17	5 09 ± 0 18

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline Each figure is derived from observations on skin samples taken from 9 adult male mice.

ment the volume of isotonic glucose solution introduced was sufficient to ensure a final concentration of 0 02 *M*, which had previously been found to give excellent results in the case of normal epidermis

The results obtained indicate the importance to mitosis of the energy *produced through the oxidation of carbohydrates. No mitotic activity* was possible in the absence of oxygen, and some apparent damage to the cells was evident. However, even in the absence of oxygen, those mitoses present in the matrix at the time the skin was separated from the body passed safely to completion. As in the case of the surface *epidermis, mitosis in the matrix* is an all-or-none reaction, and oxygen is only of critical importance in some stage preceding the visible prophase This stage has been named the antephase, and it is envisaged as a period during which sufficient energy reserves are accumulated to maintain the cell during the whole of the division.

Table II also indicates that considerable mitotic activity may develop in the absence of glucose in the medium, and it may be presumed that this activity is based on carbohydrate reserves present in the skin. Apart from these possible reserves, a fully active follicle contains a considerable quantity of glycogen, present mainly in the outer root sheath in the region surrounding the zone of keratinization. In the absence of glucose, much of this glycogen tends to disappear within 5 hours.

Experiments were next carried out to discover whether or not the concentration of 0.02 *M* glucose is in fact close to the optimum, as it is in the case of normal epidermis. Using isotonic glucose solutions the final concentration in the saline mixture was varied between 0 01 *M* and 0 06 *M*. The results of the experiment are given in Table III In contrast to the surface epidermis, the hair bulbs are able to utilize with equal facility a wide range of glucose concentration from 0 01 *M* to 0 05 *M* At a concentration of 0 06 *M* glucose, however, there were signs of mitotic inhibition In normal epidermis a similar inhibition is evident at approximately 0 03 *M* (Bullough and Johnson, 1951b).

The value of alternative carbohydrate energy sources was next tested. Various intermediates of glycolysis and of the Krebs cycle were used with the results shown in Table IV. Fructose and pyruvate provided a good energy source, although neither was any better than glucose The three Krebs cycle intermediates were far less active, a conclusion which is in general agreement with results already recorded for epidermis (Bullough, 1955a).

To obtain further evidence regarding the dependence of the mitotic activity of the hair bulb on respiration, experiments were next carried out with a variety of well-known carbohydrate inhibitors These included cyanide, which inhibits the cytochrome system and so should

be comparable in its action to nitrogen, azide, which also inhibits the cytochrome system, malonate, which interferes with the oxidation of succinate to fumarate but which, being a competitive inhibitor, ceases to have an effect when, after a few hours, sufficient succinate has accumulated, and fluoride, which is an inhibitor of glycolysis. The results shown in Table V demonstrate that all of these carbohydrate inhibitors are powerful mitotic inhibitors, and, having regard to the concentrations used, the degree of inhibition is approximately the same as that obtained with surface epidermis (Bullough and Johnson, 1951b)

TABLE III
EFFECTS OF VARIOUS CONCENTRATIONS OF GLUCOSE ON MITOSIS[a]

None	Concentrations of isotonic glucose					
	0.01 M	0 02 M	0 03 M	0 04 M	0 05 M	0.06 M
1.23 ±	6 19 ±	6 14 ±	6 25 ±	6 18 ±	6 03 ±	1 98 ±
0 27	0 22	0 38	0 24	0 34	0 49	0 30

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase Each figure is derived from observations on skin samples taken from 5 adult male mice

TABLE IV
EFFECTS OF VARIOUS CARBOHYDRATE SUBSTRATES ON MITOSIS[a]

None	Substrate 0 02 M					
	Glucose	Fructose	Pyruvate	Citrate	Succinate	Fumarate
0 84 ±	6 68 ±	5 78 ±	4 73 ±	1 79 ±	1 93 ±	0 85 ±
0 13	0 44	0 13	0 24	0 13	0 07	0 07

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase Each figure is derived from observations on skin samples taken from 5 adult male mice

Finally, the effects of 2,4-dinitrophenol were studied, and the results are shown in Table VI The action of dinitrophenol on the energy transfer systems of cells is not yet clearly defined, but the antimitotic action shown here is probably caused by an interference with aerobic phosphorylation (Loomis and Lipmann, 1948)

V Mitotic Activity *in Vivo*

From earlier studies of the mitotic activity of the surface epidermis it has been concluded that the two most important factors determining the degree of activity developing *in vivo* are nutritional and hormonal (Bullough, 1952) The effect of the nutritional factor is seen particularly

clearly in the diurnal alternation between the high mitotic activity de- [illegible]

the low activity during exercise to the closing of those capillaries. When the mitotically inactive epidermis is removed from an exercising mouse

TABLE V

EFFECTS OF CARBOHYDRATE INHIBITORS ON MITOSIS[a]

Saline alone	Saline + 0.02 M glucose	Saline + 0.02 M glucose + inhibitor		
		Cyanide		
		0.0001 M	0.0005 M	0.001 M
1.78 ± 0.02	4.64 ± 0.17	3.50 ± 0.73	0	0 (Some necrosis)
		Azide		
		0.0001 M	0.0005 M	0.001 M
		1.29 ± 0.38	0	0
		Malonate		
		0.01 M	0.02 M	0.03 M
		0.06 ± 0.05	0	0 (Slight necrosis)
		Fluoride		
		0.0001 M	0.0005 M	0.001 M
		3.42 ± 0.18	1.56 ± 0.26	0.29 ± 0.11

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is derived from observations on skin samples taken from 4 adult male mice.

TABLE VI

EFFECT OF 2,4-DINITROPHENOL ON MITOSIS[a]

Saline alone	Saline + 0.02 M glucose	Saline + 0.02 M glucose + 0.00001 M dinitrophenol	0.00005 M dinitrophenol	0.0001 M dinitrophenol
1.78 ± 0.02	4.64 ± 0.17	1.97 ± 0.32	0	0

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase. Each figure is derived from observations on skin samples taken from 4 adult male mice.

and is immersed in a saline medium with glucose and a gas phase of oxygen, mitosis recommences at once. Similarly, when the mitotically inactive epidermis is taken from a starved mouse and is placed in such a medium, mitosis recommences immediately.

No suggestion of a diurnal rhythm in the mitotic activity of the follicular matrix has been recorded, but to test the possibility that such a rhythm may exist, mice with growing hair were killed during a period of rest and after 6 hours' exercise in a revolving box. Such forced exercise in a box revolving once every 7 minutes about its horizontal axis has already been shown to reduce the mitotic activity of the surface epidermis almost to zero (Bullough, 1948). The results of these experiments are shown in Table VII, and it is clear that the behavior of the

TABLE VII

EFFECT OF EXERCISE ON MITOSIS[a]

Resting mice	Mice after 6 hours in revolving box
18 83 ± 0 52	*18 50 ± 0 49*

[a] Average numbers of mitoses arrested by colcemid in 5 hours in one hair bulb section 7 μ thick. Each figure is derived from observations on skin samples taken from 10 adult male mice

hair bulbs is different from that of the surface epidermis. Mitotic activity of the matrix cells continues at a high level, whether the animal is asleep or active, and no diurnal rhythm is present.

The effects of starvation were next studied, the mice being deprived of food but not of water for periods of 24 hours and 36 hours. After 24 hours the animals were still active, but after 36 hours they were in a state of collapse. The results listed in Table VIII show little or no effect after 24 hours, after 36 hours the mitotic activity of the hair roots was almost eliminated. Thus the mitotic activity of the matrix continues at a high level until the last stages of starvation, which is in contrast to the surface epidermis where the mitotic rate is depressed after only 12 hours' starvation (Bullough, 1949).

It was considered important to discover whether a shortage of glucose may be the only factor limiting the mitotic activity of the matrix cells of mice in the last stages of starvation. Mice were starved for 36 hours, when they were in a state of collapse, and the results are listed in Table IX. As before, the mitotic activity of the hair bulbs was greatly reduced. It should be noted that the low control figure of 1.75 was due to the fact that colcemid was not used. When skin from the two groups, control and starved, was incubated with glucose or with

fructose, a similar degree of mitotic activity was obtained This shows that the recovery of the skin taken from the starved mice was immediate and that the only nutrient lacking in the skin of these mice was the carbohydrate.

TABLE VIII

EFFECT OF STARVATION ON MITOSIS[a]

	Mice starved for	
Normal mice	24 hours	36 hours
18 83 ± 0 52	17 23 ± 0 37	1 61 ± 0 13

[a] Average numbers of mitoses arrested by colcemid in 5 hours in one hair bulb section 7 μ thick. Each figure is derived from observations on skin samples taken from 10 adult male mice

TABLE IX

RECOVERY OF MITOTIC ACTIVITY *in Vitro* AFTER STARVATION[a]

Condition of mice		Substrate	
Treatment	Mitotic rate	0 02 *M* Glucose	0 02 *M* Fructose
Normal	1 75 ± 0 09	5 12 ± 0 33	5 29 ± 0 41
Starved for 36 hours	0 68 ± 0 08	5 54 ± 0 24	5 87 ± 0.17

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick after incubation for 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase Each figure is derived from observations on skin samples taken from 5 adult male mice

The state of collapse of a starved mouse is similar in appearance to that of a shocked mouse. Shock has a powerful inhibitory effect on mitotic activity (Bullough, 1952), and the possibility was considered that the mitotic inhibition noted above was due not so much to starvation as to a condition of shock

In shock a great many physiological processes are distorted, and there is still no common agreement as to which of them is basic. However, the shock state certainly involves a profound alteration in carbohydrate metabolism (Stoner *et al*, 1950), and it was decided to test the effect of shock on the mitotic activity of the follicular matrix cells

Shock was induced in two ways In the first, tourniquets were applied to both hind limbs and were left in place for 2 hours During this period the mice remained active and in good condition, but on removal of the tourniquets shock developed gradually One hour after removal of the tourniquets colcemid was injected and was allowed to act for a further 5 hours The results shown in Table X indicate that this treatment causes a deep mitotic depression. *It is possible that the few* mitoses seen in the shocked mice all originated during the first hour

or two after the removal of the tourniquets and before the shock state was fully developed.

In the second experiment shock was induced by the injection of the sodium salt of adenosine triphosphate (ATP). The treatment given involved either a single injection of 25 mg ATP given 1 hour before the colcemid injection, or by two injections each of 25 mg ATP, the first being given 1 hour before the colcemid injection, and the second 2 hours after the colcemid injection. With only one injection, shock did not develop fully, but with two injections shock was maintained for the greater part of the 5 hours after the time of the colcemid injection. In this latter case the mice remained in a state of complete collapse and were cold to the touch. The results given in Table X show that if the shock state does not develop fully, the mitotic activity of the matrix is not greatly affected, but that in full shock, mitotic activity is almost completely eliminated.

TABLE X

EFFECTS OF SHOCK ON MITOSIS[a]

Normal mice	Mice shocked by tourniquet removal	Mice shocked by injection 25 mg ATP	
		1 Injection	2 Injections
18.83 ± 0.52	4.17 ± 0.82	15.63 ± 0.70	0.76 ± 0.03

[a] Average numbers of mitoses arrested by colcemid in 5 hours in one hair bulb section 7 μ thick Each figure is derived from observations on skin samples taken from 10 adult male mice

In a further comparison between the effects of starvation and of shock, the possibility of a mitotic recovery after a shock inhibition was considered. Shock was induced as before both by the removal of tourniquets and by two injections of ATP, and small pieces of skin containing hair bulbs in which mitosis was inhibited were cultured in a phosphate-buffered saline medium with glucose and an oxygen gas phase The results listed in Table XI show that the hair bulbs from both groups of shocked mice made an immediate recovery when the skin was separated from the body and immersed in the saline medium with glucose

VI. *Discussion*

There are at least three critical processes in the production of a new hair. First, there is the formation by mitosis of the mass on new cells, second, there is the orientation of these cells into concentric sheaths, and third, there is the process of keratinization whereby the hair itself is formed Ryder's evidence, derived from a study of the absorption of radioactive materials, suggests that glucose enters rapidly and selectively

into the matrix cells, while cystine is more readily absorbed in the region of keratinization. This provides corroborative evidence that the great activity of the matrix cells results in a high demand for glucose as the raw material for respiration In addition, the matrix must also make a high demand for all those other substances which form the structural basis of the new cells. However, it would seem that, as in the case of the surface epidermis (Bullough, 1952), these other substances are never normally in short supply, even in the hair bulbs of a mouse which has collapsed from starvation

TABLE XI
RECOVERY OF MITOTIC ACTIVITY *in Vitro* AFTER SHOCK TREATMENT[a]

Condition of mice		Substrate	
Treatment	Mitotic rate	0 02 *M* Glucose	0 02 *M* Fructose
Normal	1 75 ± 0 09	5 62 ± 0 38	6 66 ± 0 52
Shock by tourniquet removal	0 84 ± 0 09	6 80 ± 0 34	6 47 ± 0 59
Shock by injection 2 × 25 mg ATP	0 69 ± 0 13	6 29 ± 0 23	6 28 ± 0.30

[a] Average numbers of mitoses arrested by colcemid in one hair bulb section 7 μ thick in 4 hours at 38°C in phosphate-buffered saline and an oxygen gas phase Each figure is derived from observations on skin taken from 5 adult male mice

Such a highly active system as a growing hair follicle would be impossible unless it had access to an excellent blood supply As a follicle begins to elongate there is an apparent heavy deposition of fat in the hypodermis This fat is probably inert and it may perhaps have little function except to provide the space in which the elongation can occur. However, with the deposition of this fat, the capillary network of the skin becomes more obvious, and the impression may be gained that new capillaries have been formed (Durward and Rudall, 1949) However, work now being done by Elsa M Firth (personal communication) suggests that this is not the case Rather it seems that the already existing capillary network is stretched out, and that possibly more blood passes through it. Firth has shown that in the mouse the dermal and hypodermal capillaries do not form close networks around the follicles, and that furthermore there is no blood supply to the dermal papillae. In the rat (Durward and Rudall, 1949), the stouter follicles are closely ensheathed in a basketlike network of capillaries, while the largest follicles of all, those of the vibrissae, are enclosed in a blood sinus Even in these

cases, however, the dermal papillae are relatively lightly vascularized Evidently, in all these cases, the critical supplies of glucose and of other raw materials reach the matrix cells not through the dermal papilla but through the outer surface of the bulb.

However, the most fascinating problem concerns the nature of that stimulus whereby the quiescent follicle bulb is forced into high activity. This stimulus is not nervous, and is not caused by the diffusion of some substance from follicle to follicle (Durward and Rudall, 1949). To this it may be added that the stimulus most probably does not emanate from the capillary system. A plausible hypothesis would seem to be that the control of growth resides in the dermal papilla, which in the mouse may be supposed to resume its activity at approximately monthly intervals. On this hypothesis, the stimulus from the papilla has the primary effect of inducing active mitosis in the matrix, and the secondary effect of inducing a heavy fat deposition and possibly an increased blood flow in the dermis. In the mouse, follicular reactivation passes like a series of recurrent waves over the body surface, while in the sheep and on the human head each follicle operates as an independent unit. The really fundamental problem yet to be solved concerns the nature of the recurrent stimulus to active mitosis, whether in whole areas or in single follicles.

VII. Summary

1 The distribution of mitotic activity in the growing follicle of the adult mouse is described In the fully grown follicle such activity is confined to the matrix cells of the bulb

2. Observations *in vitro*

(a) It is shown that, for the development of active mitosis in a hair bulb, adequate supplies of oxygen and of some suitable carbohydrate substrate are essential In the absence of either, the mitotic activity is powerfully inhibited

(b) Ideal carbohydrate substrates for the support of mitotic activity are glucose, fructose, and pyruvate The various Krebs cycle intermediates tested were not efficient in this respect.

(c) Any substance which is known to inhibit the process of glycolysis, of the Krebs cycle, or of the cytochrome system also inhibits the mitotic activity of hair bulbs The substance 2,4-dinitrophenol which is said to inhibit the process of energy transfer has the same effect

(d) All the available evidence therefore points to the conclusion that the high mitotic activity of a hair bulb can only be maintained by a high level of energy production in the cells. Therefore it must be expected that mitotically active hair bulbs will normally absorb large

quantities of glucose and oxygen, and this is supported by the observations of Ryder on the rate of uptake of radioactive glucose.

3. Observations *in vivo*.

(a) Unlike the surface epidermis the matrix cells of a rapidly growing hair follicle show no signs of any diurnal rhythm. No mitotic depression is seen after 6 hours of forced exercise.

(b) In starved mice the mitotic activity of the matrix cells does not become depressed until the animals are in a state of collapse after about 36 hours, and at that time the addition of glucose is all that is needed to restore the mitotic activity to normal

(c) In full shock induced by the removal of tourniquets or by the injection of ATP the mitotic activity of the matrix cells is almost completely inhibited, but in partial shock mitotic activity is not greatly affected. In skin taken from fully shocked mice and incubated with glucose, mitotic activity returns to normal almost immediately.

VIII. Acknowledgments

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CHAPTER 9

The Vascularity and Patterns of Growth of Hair Follicles

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I. INTRODUCTION

A. Historical Note

Our knowledge of the blood supply to the skin and its appendages is based principally on observations by German workers during the latter half of the last century. Their studies were concerned with two main aspects, the blood plexuses of the "whole" skin and the special

blood supplies to hair follicles, sweat glands, sebaceous glands, and arrector pili muscle. Unna (1908) summarizes all these observations, some of which will be recalled here. Studies of the vascular system of catagen-telogen follicles were incidental to other problems in the work of Stieda (1910), Segall (1918) and Merkel (1919).

Comprehensive accounts of the vascular supply to the whole human skin have been reported by Spalteholz (1893, 1927), Bellocq (1925), and Salmon (1936). Spalteholz (1893) described the vascular system in terms of three well-defined arterial plexuses; fascial, cutaneous, and subpapillary. The deepest, lying in the superficial fascia, connects together the arteries from the body wall to give a wide meshed net—the fascial plexus—from which branches pass to the subcutaneous fat. Branches from the arteries in the subcutis pass upward to reach the zone of junction between the cutis and subcutis where they form another plexus, the cutaneous plexus. The vessels of this net are not, however, strictly in one parallel layer but frequently project beyond and are embedded in the undermost layer of the more densely fibrous corium. Again, from the cutaneous net fine branches pass toward the dermal papillae and in the outer half of the corium give a narrow meshed subpapillary plexus. In turn this plexus sends single loops into the dermal papillae beneath the epidermis, or, where papillae are absent or inconspicuous, a network of fine vessels is formed close beneath the epidermis (as in human head skin). Spalteholz (1927) gives an important list of English and French synonyms as follows:

cutane Netz: subcutaneous plexus, réseau sous-dermique oder hypodermique oder profond
subpapillaren Netz. subpapillary plexus, réseau sous papillaire oder sus-dermique oder dermique

A fourth arterial plexus, found in certain places, is especially characteristic of the human scalp. This, Spalteholz calls the "sub-cutane Netz," which may give rise to confusion if the above list of synonyms were strictly adhered to. This "sub-cutane Netz," which we will refer to as "subcutaneous plexus," lies in the subcutis and on the musculo-aponeurotic layer. We wish to emphasize its existence for it is so similar to that occurring in the skin of various animals. The above study of the vascularization of human skin has provided a basic plan to which students of animal skin have attempted to relate their findings. However, the terms used are not always clear and this derives from the unnecessary multiplicity of terms for skin structures.

B. A Criticism of Terminology

We will diverge here to seek a simplification of the terminology. Koelliker (1889) gives a beautifully measured description of the structure of skin with easy and clear definition of his terms. In this, Lederhaut (corium) and Unterhautbindegewebe (stratum subcutaneum) are the two main divisions of the cutis or dermis. Hoepke (1927), however, abandons all terms except "corium" and prefers that this include the stratum adiposum. There is, of course, wide usage of terms like cutis and subcutis, dermis and hypodermis, stratum adiposum, subcutaneous fat and panniculus adiposus. Since the one universally agreed term in the structure of skin is the epidermis, there seems no case for any other term than dermis for the underlying tissue. The word dermis has its origin in the act of flaying and thus refers specifically to the skins or hides of animals. The root has been so widely used for other important purposes, ecto-, meso-, endoderm, that there is undoubted restriction in the employment of Greek prefixes

In this article we shall think in terms of three superposed layers, epidermis, dermis, and hypodermis. The epidermis has its stratified layers of epithelial cells and the lower strata transform into the upper The dermis has its layers too, but these, of course, are not changing the one into the other. They are relatively fixed parts of a whole and consist of a subepithelial pars papillaris, the pars reticularis and perhaps in thicker skins a pars fasciculi, to adopt the term suggested by Proctor (1922) Together these fibrous layers are the equivalent of the corium as defined by Koelliker, but we should regard the combined pars papillaris and pars reticularis as the irreducible minimum structure of the thinnest dermis and in that sense the really characteristic parts of the dermis

Underneath the dermis there is tissue of a much less densely fibrous nature which may or may not be abundantly filled out with fat cells, but is potentially fatty Stratification often occurs, there being layers which have the character of superficial fascia, some which are compact masses of fat cells and others such as the striated muscle of the panniculus carnosus with its associated epimysium above and below We cannot refer to all this tissue as the hypodermis but have restricted the term to that layer of tissue in between the densely fibrous dermis and the panniculus carnosus Tissue below the skin muscle, between it and the body wall, we call subcutaneous tissue. We refer to the several concentrations of fatty tissue within the skin as follows If there is a distinct fatty layer within the dermis, such as often occurs in sheep skin between the pars reticularis and pars fasciculi, then we refer to this as the dermal fat A fatty layer just beneath the dermis we refer

to as the hypodermal fat, while fatty deposition in the superficial fascia beneath the panniculus carnosus we refer to as subcutaneous fat.

C. *Vascular Supply of Skin and Its Derivatives*

The above diversion to explain our terminology is necessary so that we can describe the relationships of the blood plexuses in the skin of man and animals The terms used by Spalteholz do not suit our terminology, but those used by French workers are directly applicable. For example, the subpapillary and cutaneous nets become dermal and hypodermal, respectively, in French usage. In the general case we believe there is also a subepidermal plexus above the dermal plexus which becomes more obvious in the skins of larger animals. The outermost subepidermal-dermal plexuses are the important ultimate supply to the growing epidermis and the appendages of the skin, while the hypodermal plexus is more in the nature of a conducting network to bring about even distribution to the metabolically important plexuses above.

Bellocq (1925) has distinguished in human skin three kinds of supply involving the dermal and hypodermal networks.

1 *Independence of Areas*

Neighboring areas are isolated from one another and the dermal and hypodermal anastomoses occur only within each area and do not spread uniformly throughout the skin It is frequently met with in the newborn, in our studies of an advanced sheep fetus a situation of this nature was very obvious. This independence of areas in very young animals is looked upon as due to incomplete development of the vessels, in the aged it is regarded as being due to the senile degeneration of skin arterioles Without greater experience with the material, we are not entirely convinced of the reality of Bellocq's concept of independent areas vagaries resulting from incomplete or irregular injection might play some part in producing an appearance of independence Spalteholz (1927) does not believe that apparent isolation of areas is due to lack of anastomoses between them

2. *Dermal Anastomoses Only*

This is the situation where there are fine anastomoses in the dermal plexus and none at the hypodermal level It is encountered in man chiefly in the dorsal and ventral regions of the hand and foot where hypodermal arteries are particularly numerous Bellocq remarks that this assures the supply to the metabolically active epidermal-dermal region

3 The Double Plexus

This is the classical type of Spalteholz where widespread anastomoses occur at both dermal and hypodermal levels.

In man the disposition of these plexuses (1–3 above), and their variations in different body regions and with age, have been carefully studied. It is said, concerning the skin of the head, that with age the anastomoses of both dermal and hypodermal plexuses decrease progressively so that ultimately some areas of the skin are poorly supplied with blood (Hoepke, 1927).

Such knowledge of the skin circulation is important in understanding the varying supply to special skin structures, such as the fat lobes or groups of fat cells, the hair follicles, sebaceous glands, arrectores pilorum muscles and sweat glands. Koelliker (1889) describes the situation where hair follicles are sparse, the fat lobes are discrete and well developed, and the sweat glands are not crowded. In this case he is able to speak of the capillary supply to each as being quite separate. The supplies arise in series from a principal artery, small branches of which go first to the fat lobes, higher up to the sweat gland and finally at the level of the sebaceous gland there is a recurrent branch to the follicle. All these branches give rise to capillary nets of which those to the fat lobes are so rich that often each fat cell lies in a separate capillary mesh. The next richest supply is to the follicle wall, from the lowest regions of which a loop passes into the hair papilla, while sparse branches from the follicle wall network go to the sebaceous gland and the arrector pili muscle. The sweat gland in man receives much more blood than the sebaceous gland.

Unna (1908) reviews earlier work as well as describing his own studies. He recalls that Tomsa (1873) found, especially in man, that the commonest arrangement was for the follicle to be supplied at the sebaceous gland level by a recurrent branch from the dermal plexus. From his own work he finds, in man as well as in all other animals studied by him, that the supply was often from the hypodermal plexus below. This may occur from a large vessel which passes beneath the follicle giving branches into the papilla and to the follicle wall. The network on the surface of the follicle had been described previously (Koelliker, 1889) as consisting of longitudinal arterial capillaries which give off mostly horizontal cross-connecting branches, likened to a ladder. Unna prefers the picture of an irregular rhombic lattice work with no marked longitudinal elements. From the lowest loop of the follicle network there arises a loop into the hair papilla and this is described as being better developed in animals than in man. The follicle network may be so closely meshed as to cover the follicle completely, or again

it may be quite open and most irregular. Where Unna's observations were made on fetal and newborn material he did not press the general applicability of his findings.

Spalteholz (1927) summarizes his views as follows: the appendages of the skin, being derived from the epidermis, get their arteries at an early stage from the subpapillary net, just as do the papillae protruding into the epidermis. As the hair bulbs and sweat glands grow into the subcutis, they come into closer contact with vessels arising in the subcutis or from the cutaneous net. Finally there is, for the larger hairs and sweat glands, a twofold vascular supply—the upper parts derive their arterial blood from the subpapillary net, the lower parts from the deeper vessels.

Durward and Rudall (1949) described especially dense capillary networks in the lower half of monotrich follicles in the rat Awl or auchene follicles were shown to have less dense capillary networks while *the smaller follicles (zigzags) had an inconspicuous supply. These observations* suggested a relation between the follicle wall circulation and the amount of fiber substance being produced. Furthermore, the resting follicles were still supplied by vessels arising from the lower regions of the skin. Goodall and Yang (1954), by means of diagrams, report on the supply to the skin appendages in 3-month-old calves, where the follicles are shorter and of less diameter than the largest we have illustrated for the rat skin. The networks to the follicle wall are considerably less conspicuous than in the case of rat monotrich follicles or many awl-producing follicles However, the papillae in the calf skin follicles have a rich network of vessels. The circulation to the sweat glands is poorly developed, while that to the sebaceous glands is by contrast, most abundant Goodall and Yang summarize the origin of the various capillary supplies as follows: the sebaceous glands are supplied from the uppermost plexus (= subepidermal) while the supply to the lower follicle wall is quite separate from that to the papilla, but both supplies arise from the middle plexus (= dermal). Curiously, the meager supply to the sweat gland is figured as arising from the vessels which go directly to the hair papilla Ryder (1955b) describes the circulation to different follicle types in many breeds of sheep and in embryos; his paper should be consulted if detail is required. In the papilla there may be a single loop in small follicles or a well-developed network in larger follicles. The papilla supply generally arises from the network of capillaries on the follicle wall, occasionally, especially in larger follicles, the papilla may be supplied from the deepest plexus (hypodermal), from the middle plexus (dermal), or from vertical vessels joining these two networks Ryder also gives descriptions of the less prominent cir-

culation around the upper part of the follicle and of that to the sebaceous glands, both arising from the uppermost or subepidermal plexus. The sweat glands are poorly supplied with capillaries as in cattle skin

In summary we can say that the upper parts of the follicle and the sebaceous glands receive their supply from the subepidermal and dermal plexus, the main follicle supply comes from either the dermal or hypodermal plexus or from vertical vessels connecting these two, the supply to the papilla may be just a loop or network arising from the follicle wall circulation, or it may be quite separate. Just where the supply comes from might be of importance to the functioning of the follicle in cases of superficial injury, or where there are marked changes in external temperature, or again, where there are depositions of fat at a certain level in the skin

It must be remembered that follicles can develop and grow without a blood supply, as is shown by tissue culture techniques (Hardy, 1951), however, they then produce smaller hairs. In the intact skin the blood supply to the follicle clearly varies according to the size of the follicle and the type of fiber being produced. There is a lesser supply to the upper part of the follicle (excepting the rich supply to the sebaceous glands) The major supply is to the lower half or third of the follicle or it may even be concentrated over a comparatively small zone just above the bulb of the follicle The follicle may have a rich circulation to its wall, with or without a circulation to the papilla Or again, the vessels in the papilla may be relatively well developed, while those of the follicle wall may be relatively poorly developed. All these various arrangements may not be without significance in relation to the kind of fiber being produced and the rate of its production. There is always the possibility that they may have a meaning with reference to factors or structural features we have as yet failed to recognize

II. Vascular Patterns in Rat Skin

A. *The Vascular Plexuses*

We have concerned ourselves only with the main areas such as back, flank, and ventrolateral regions where frequently there are longitudinally disposed bands of hair growth, which move from venter to dorsum and exhibit the standard range of pelage fiber types, monotrichs, awls, auchenes, and zigzags (Dry, 1926, 1928) The animals, mostly in the third or fourth hair generation, are of an ideal size for injecting the vascular system, only albino types have been used to avoid possible confusion between pigment and the India ink injection

Where we have wished to study the vascular system associated with the growth waves we have cut our sections in the dorsoventral plane

across a band of growing follicles and parallel to the slope of the hairs. In this way any planar net of blood vessels then appears to be a broader band as indicated diagrammatically in Fig 1. The distribution of blood vessels is well shown in Fig. 2, which is such a section at a dorsolateral

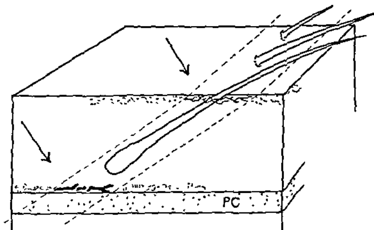


FIG. 1 Diagram to explain plane of section If the slice of tissue indicated by the broken lines is viewed from the position of the arrows then vascular plexuses appear as extended networks rather than as cross sections of planar plexuses PC = Panniculus carnosus

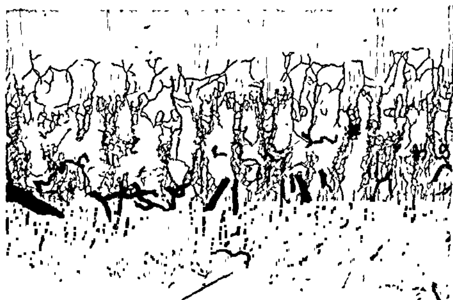


FIG 2. Rat Quiescent dorsolateral skin region immediately in front of advancing wave Magnification × 29

region containing quiescent follicles The obliquely cut section shows the longitudinally disposed muscle fibers of the panniculus carnosus as columns lying perpendicularly to the skin surface, these being emphasized by fine capillaries between the muscle fibers. Below the skin muscle there are portions of a planar fascial plexus. The greatest vessels of the skin lie immediately above the panniculus carnosus as a well-developed wide-meshed plexus, there being superposed venous and arterial networks. The next most easily distinguished plexus lies at the

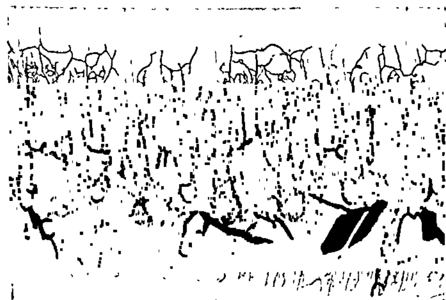


FIG 3 Rat Early active skin just ventral to Fig 2 Magnification × 29

outer surface just beneath the epidermis and consists of a moderately fine meshed net of small caliber vessels This net is connected to the main plexus on the panniculus carnosus by fairly prominent vessels and as the skin is contracted in thickness due to the quiescent state of hair growth, these vessels are not straightened out but have a somewhat sinuous course, especially in the deeper regions Between epidermis and muscle there is much vascular tissue so concentrated about the sebaceous glands as to suggest a fine plexus at that level too Many small vessels occur around the bases of the group of resting follicles from which fine sinuous cross-connected vessels lead to the main plexus below.

More ventrally than seen in Fig 2, the follicles have grown downward and Fig 3 shows the stage where fiber tips have formed and

nearly reach the surface of the skin (this is the third generation of hairs). The skin is a little thicker, other changes which have occurred indicate that as the follicle grows downward it causes the expansion of the persisting but condensed leash of vessels which remained below the resting follicles. Further ventrally the follicles are still more developed and hairs have penetrated through the skin to a distance of a few millimeters (Fig. 4) The skin is now much thicker, while the capillary net-

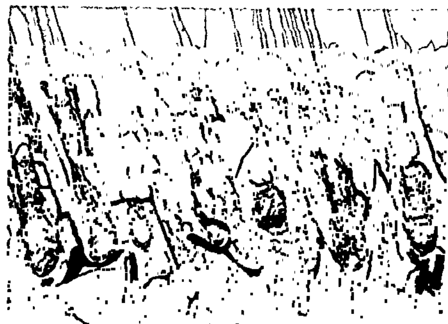


Fig 4 Rat More fully active skin ventral to Fig 3 Magnification × 29

works about the follicles have increased, several awl-type follicles are illustrated and are of greater diameter than those of Fig 3. While one follicle in Fig. 4 still shows mainly horizontal capillary elements, presumably due to the rapid increase in the diameter of the follicle base, from now on this increases little and the capillary system finally adopts a form best described as an irregular rhombic network. Figure 4 also shows the stouter nature of the vessels connecting the subepidermal plexus to the main supply plexus on the panniculus carnosus and such vessels are also straighter than those shown in Fig. 2, being perhaps extended as the skin increased in thickness In Figs. 2 to 4 there is an increase in the capillaries at the level of the hypodermic fat.

B Hair Growth Waves and Associated Vascular Changes

1. *Telogen-Anagen Areas*

The progress of waves of growth in the rat often proceeds by pulses in that the hair at one level having penetrated the surface, the zone just dorsalward is not covered for many days. Thus material will be obtained, either just before the new pulse occurs, or at some stage perhaps just after the pulse has occurred This latter stage is represented in Figs 2 to 4. The former stage is illustrated from another rat in Fig. 5 and an abrupt transition is very obvious in the thickness of the skin



Fig 5 Rat Sudden transition zone from quiescent (left) to active (right). Magnification. × 17.

and in the follicle lengths. Figure 5 also shows the very rich blood supply to the sebaceous glands in this animal, and that there is no clear distinction to be made between the supply to glands in the quiescent zone and to those at least just inside the active zone Well inside the active zone we have observed the increased caliber and comparatively straight course of the vessels joining the subepidermal plexus with that of the panniculus carnosus.

2 *Catagen-Telogen Areas*

At the receding edge of the growth wave the follicles shorten below the level of the sebaceous glands and the dermis and hypodermis be-

come thinner so that the plexus on the panniculus carnosus remains about the same distance from the follicle base. The vessels in the lower follicle wall become greatly compressed together by the contraction in follicle length, but there is little change in the diameter of the lower follicle. The dermis is now near its minimum thickness and the next step consists in the rising of the hair club toward the surface leaving behind the network of capillaries as a tube. This collapses and, with degeneration of some of the capillaries, appears as a system of fine wavy vessels. These processes can be followed in Fig. 6 where (a) the section



FIG 6 Rat Vascular changes associated with hair club formation Magnification × 38

just cuts into the side of a follicle contracted in length, but whose base is still deeply placed in the dermis, (b) a hair club is forming and the base of the follicle has ascended in the dermis; at (c) the fully formed club has moved near to the surface leaving behind a collapsed network, while (d) represents the final stage adopted by the vessels beneath resting follicles Figure 6 also gives a more comprehensive view of the blood plexuses We see now a better view of the planar plexus below the panniculus carnosus and the vascularization of the subcutaneous

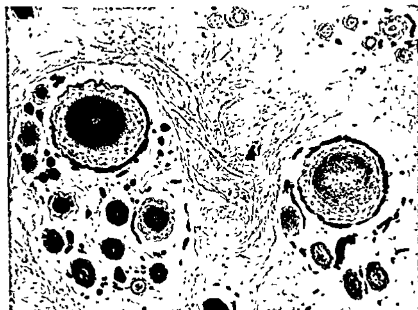


FIG 7 Rat Variation in blood supply to different follicle types Magnification, × 120

fat. Also the complex net of vessels at the sebaceous gland level is most evident

We will not illustrate here the features associated with those regions lacking an underlying panniculus carnosus. In a previous paper (Durward and Rudall, 1949), Figs 7, 8, and 10 were from such a region, namely, laterally and ventrolaterally about the level of the forelimb For our purpose on that occasion, it was a good choice, as the follicles are very straight, not being deflected into "golf club" shapes by pressing against a panniculus carnosus. The main plexus of vessels is not restrained by attachment to the muscle layer and occupies in Fig 9 (Durward and Rudall, 1949) the level between dermis and hypodermis—a typical hypoderma

C. The Vascular Supply to Different Follicle Types

It is immediately obvious from Fig. 4 (and from Fig. 7, Durward and Rudall, 1949) that only the largest follicles have a pronounced basketwork of capillaries around them, and this varies somewhat from follicle to follicle. There is a sharp distinction between the supply to monotrichs and awl-type follicles on the one hand, and to zigzags on the other. In the former there is a close investment by a fine-meshed network of capillaries while this is lacking in the case of zigzag types where the blood supply consists of mainly longitudinal fine vessels in the fatty tissue filling the spaces within the follicle group. This plan is very evident in the cross section shown in Fig. 7. The section is made below the level of keratinization and shows a larger group containing a monotrich follicle and a smaller group containing an awl follicle. Both the large follicles have a closely adhering investment of capillaries lying just outside the circular layer of connective tissue fibers covering the outer root sheath. The smaller follicles (zigzags) have no such investment, instead, near them are a number of injected vessels lying parallel to the follicles and seen cut transversely.

In general terms we can see that the volume/surface relationship of the larger follicles is very much greater in the monotrich and awl follicles than in zigzags and probably to a great extent this accounts for the differences in the vascular supply. Furthermore, it may well be that the fibrous layer covering the outer root sheath is thicker or more compact in larger follicles. These are the kinds of structural features we should have to consider in assessing the significance of variation in vascularization with follicle type.

Between monotrich and awl-type follicles there are some pronounced differences. In general the capillary network on a monotrich follicle is closer meshed, and is composed mainly of horizontal elements above the bulb, while on the bulb itself these form a more irregular mosaic (Fig. 6, Durward and Rudall, 1949). In the fully developed awl follicle the mosaic pattern is more general over the whole lower half or third of the follicle. However, a major difference occurs in that we regularly find the papilla vessels injected in monotrich follicles, while we have not found injected papillae in awl-type follicles. The principal features of the vascular supply to a monotrich are shown in the serial sections of Fig. 8. Each section is approximately 50 μ thick, either there are very numerous capillaries within this papilla or the injection has caused distension of the individual vessels thus making intercapillary space invisible, there is no evidence of leakage.

Thus the vascular supplies to monotrich and awl follicles differ in

important respects This situation is a crucial one in forming views on some aspects of the relationship of vascularization and follicle activity. As fibers, monotrichs and awls differ in many ways. The monotrich is nearly circular in cross section, while the awl is shaped as a bean or often approaches the dumbbell shape of rabbit guard hairs. Associated with this, the papilla is more circular in cross section in monotrichs and



Fig 8 Rat Series of sections through injected monotrich follicle. Magnification × 27.

oval or much flattened in awl follicles, the particular shape of fiber cross section being determined in the same general way as we have described for the rabbit (Durward and Rudall, 1955) More important differences concern the ratio of fiber diameter and rate of growth in length At present we lack sufficient detailed measurements, but it is possible that for equal-sized follicles and papillae, the monotrich can produce inner root sheath and fiber material at a greater rate. We may be able in time to associate a papilla circulation with an increased rate of cell division

In a natural growth wave monotrich follicles are a little precocious in restarting, Fig. 9 gives a view of a monotrich which is more ad-

vanced than any of the other follicle types surrounding it. The resting monotrich follicle would appear to have a greater supply of capillaries and this might help to reduce the length of the resting period While the monotrich is a little precocious in recommencing growth, it is more remarkable for continuing to grow for many days after the other hair types have ceased growing. Such a fact is readily seen when



Fig 9 Rat Monotrich follicle in early activity Magnification × 38

the hair is clipped, say on a dorsal or dorsolateral region, at a time when most of the fibers have ceased growing Then, seemingly, only the monotrichs continue and may reach lengths of ½–1 cm While it is not possible to say that the possession of a papilla circulation is the direct cause of this prolonged growth, it is necessary to say that these two features go together

We recall some relevant facts from Dry's great studies of hair growth in mouse and rat. Concerning the second hair generation in the rat he says· "the larger hairs finish growing somewhat later than the smaller

ones, the monotrichs being especially late in becoming club hairs. At the beginning of the growth of G_2, the bigger hairs likewise have an advantage over their smaller neighbors in a slightly earlier start. . . . In the second generation of the mouse these differences in commencing and completing growth are not found" (Dry, 1928).

We might take the view that all these phenomena are inherent in the particular follicles and have a primary cause which we are not likely to see under the microscope Nevertheless, there are many features of the vascular system which would seem to give a reasonably adequate explanation of these phenomena

Rat (G_3 and G_4 generations). Larger follicles, when resting, have a richer vascular supply to their bases than smaller follicles The larger follicles begin to grow before the smaller ones

Monotrich follicles possess a vascularized papilla They continue to grow much longer than other fiber types.

Mouse Hardy (1952) gives some information on the blood supply to hair follicles in the adult mouse. Her illustration (Plate, I, Fig 11) does not depict anything like the rich blood supply to active follicles in the rat. Relevant to our present argument she makes this important observation. "No special blood supply to the cells of the new follicle roots was observed in the stages prior to hair formation." This situation is quite different from that occurring in the rat. Therefore, the lack of differences in the time at which various follicle types in the mouse recommence growth can be associated with the lack of outstanding vascular supplies to the bases of resting follicles.

Furthermore, Hardy states: "In the mouse no blood vessels were seen entering the dermal papillae at any stage of the growth cycle, although a careful search was made in serial sections and whole mounts." Thus, if in the rat we can explain the delayed catagen-telogen phase as due to the possession of a vascularized papilla, then the absence of persisting growth in mouse monotrichs is equally explained by the lack of a vascularized papilla

The purpose of these arguments is to emphasize those points where the type of blood supply may affect fiber growth and the hair growth cycles For the rat these points are as listed below:

1. Follicles producing the largest hairs have the largest blood supply and this increases in extent from the time the resting follicle extends into the hypodermis until the maximum output of fiber is achieved.
2. Resting follicles which have a richer supply of blood capillaries recommence growth earlier
3 Follicles which have a papilla circulation are unusually prolonged in their growth period

III. Vascular Patterns in Rabbit Skin

A. The Vascular Plexuses

In rabbit skin the study of vascular patterns is made easier by the absence of hypodermic fat and by the relatively feeble development of sebaceous glands. The phenomenon of limited bands of growth, moving over the skin surface as a wave, is not so regularly encountered as in the rat, but in our animals we frequently found these longitudinal bands which moved from dorsum to venter, just as described by Hale (1945).

In the main dorsal and lateral areas behind the shoulder, the principal vessels supplying the skin consist of large arteries and veins lying on the upper surface of the panniculus carnosus, supplying the muscle

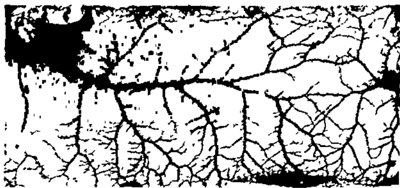


Fig 10 Rabbit System of vessels on superficial face of panniculus carnosus Magnification × ½.

itself and giving finer branches to fascia below the muscle The panniculus carnosus of the rabbit is very easily stripped off as a layer and a portion from the anterior lateral region is shown in Fig 10. The main vessels arise from deeper levels anteriorly and large veins and arteries pass backward closely parallel to one another. There are frequent anastomoses within each system, both between large vessels and small vessels In type this plexus is very similar to the subcutaneous plexus of human scalp, as described and figured by Spalteholz (1927)

As in the rat, we have cut sections across the wave front, that is in a dorsoventral plane, and parallel to the hair slope Figure 11 gives a view at the advancing edge of the hair growth wave At the bottom of the section, the panniculus carnosus is seen with the superimposed main "subcutaneous plexus." Above this is a clear zone of loose connective tissue, which in the rat and guinea pig is occupied by fat cells Above this hypodermal region in the underlayers of the dermis, there is an open network of finer vessels—the equivalent of a very poorly de-

veloped hypodermal plexus. In the upper levels of the dermis, stouter vessels represent portions of the dermal plexus; these are better illustrated in Figs. 12 and 13. Just beneath the epidermis are the much finer vessels of the well-developed subepidermal plexus The main regions of the dermis are seemingly without capillaries and smaller vessels. Large vessels pass from the subcutaneous plexus to the dermal plexus, being connected by smaller side branches to the hypodermal plexus as they enter the dermis from below.



Fig 11. Rabbit. Junction of quiescent zone and newly active zone of hair growth Magnification × 23

B Hair Growth Waves and Associated Vascular Changes

1. *Telogen-Anagen Areas*

This particular rabbit was killed and injected when one pulse of growth was well established, while the next pulse of growth had not as yet taken place. The obviously growing follicles to the right of Fig 11 possess fibers just penetrating through the skin while those immediately to the left are quiescent. The follicles are in the characteristic groups and each group has its own separate vascular system. In the growing follicles stout vessels lie between the follicles of a group and parallel with them and are cross-connected by a few fine vessels. By contrast, the resting follicle group has beneath it a characteristic leash of fine vessels of seemingly uniform caliber, combining into larger

vessels below, and having connections with the dermal plexus above and with the hypodermal plexus below. It is clear from the illustration that as the skin thickens due to the increasing length of neighboring growing follicles, the wavy leashes of vessels become stretched out

We have noted that the wave bands often progress as a pulse, but on other occasions they seem to progress continuously but slowly. Figure 11 suggests that a movement of the latter kind might be due to regrowth being stimulated by the mechanical extension of the vessel system of those follicle groups next to newly active follicles that are lengthening

Well within the growing zone, the follicles are longer than those at the advancing edge of the wave (Fig. 11) and the dermis in consequence appears thicker. The hypodermis or clear zone between dermis and skin muscle remains the same thickness; there is no deposition of fat associated with hair growth in these rabbits. The main vessels to the lower half of the follicle are connected to the dermal and hypodermal plexuses

2. *Catagen-Telogen Areas*

At the receding edge of the growth wave we see the relatively sudden transition from fully growing follicles to catagen and telogen follicles. There are a number of important features in Fig 12, which are described below.

(a) and (b). Two groups containing growing follicles and of full length The groups have characteristic small vessels joining them to the hypodermal plexus.

(c) The section has caught an isolated follicle in the process of club formation, which is seen at the level of c, having ascended nearly to a mid-dermal level Stretched out below this club is a tight leash of vessels passing down toward the base of the dermis

(d) A group in which there is one large central follicle and many smaller laterals, all still growing and without signs of club formation. The follicle lengths have diminished and with this there is much waviness of the finer vessels on the lower quarter of the follicle As the follicles contract in length the dermis becomes thinner.

(e) *The condition of the vessels is typical of that in catagen and* early telogen follicles Only the "apex" of a conical leash of vessels appears in the section. Although this group is attached to that of d, both at the base, and laterally by stouter vessels, the fibers of e are in a distinctly later stage than those of d

(f) Only finer "fur" fibers are present in the visible part of this group and their bases are at about a mid-dermal level

(g) Club hairs have formed and have ascended to their final level.

(h) Portions of dermal plexus as it commonly appears within growing regions.

(i) Portions of dermal plexus more characteristic of quiescent zones.

In both Figs. 12 and 13 the hypodermis and panniculus carnosus were lost during manipulation and sectioning. The bottom surface of the sections is a natural cleavage plane. The dermis to the left in



FIG. 12 Rabbit. Transition zone from active (left) to quiescent (right) Magnification. × 18

Fig. 12 is the maximum thickness, but to the right it steadily decreases in thickness, corresponding at first to diminishing length of the follicles, but the process continues thereafter, even though the club hairs have formed and reached their proper level During this process the vessel systems below the resting follicles remain especially conspicuous. In Fig 13 some features associated with this decrease in skin thickness are illustrated, as well as others of interest

1. A hair club of a larger fiber is in process of formation at the level of *1* and has been "pulled" through the mainly parallel vessels which formerly surrounded the lower part of the follicle. These are visible extending from the bottom of the club toward the hypodermis A remarkable feature of this hair group is that the smaller fibers still

extend to the bottom of the group, and there is no sign of club formation in them.

2. In this group the largest fiber has formed its club and has ascended to about its final level. All the smaller fibers of the group still grow from follicle bulbs at the base of the group, there being no sign of club formation.

3. The plane of sectioning cuts obliquely through the group, but the bottoms of the finer fibers are visible at the level of 3 and there is no evidence of club formation.



Fig 13 Rabbit Changes in the vascular pattern with the development of the resting stage Magnification × 23

4 The largest fiber of this group is a club hair and has ascended to its final level, but very many fine fibers still extend deeply to the level of *4*. The vessels in *4* are still moderately straight compared with the condition in *5* and *6* where there is increasing waviness as the dermis contracts in width.

5. This group and the two groups immediately to the right of it still contain some fine fibers deeply extending to about the mid dermal level.

6 In the series of groups from left to right, this is the first in which it can be said that all the fibers are club hairs and have ascended to their final level

Attention is drawn to the interconnections between vessel bundles seen at the bottom right of Fig 13. They raise the question as to what

complex "circuits" exist within and between follicle groups It is just conceivable that coordination of growth as represented by the growth wave phenomena may depend on special vascular circuits.

The rabbit provides outstandingly clear pictures of the vascular changes in relation to hair growth. Compared with the rat this is due to the absence of fat cells in the hypodermis, while the follicles at their greatest length merely stretch the dermis without penetrating through it The greater degree of vascularization within the growth wave, as compared with that in quiescent zones, is caused in part by the numerous stout vessels associated with the very elongated active follicles In addition, the vessels of the dermal plexus are clearly enlarged in caliber as is suggested by Fig 12, but this is more readily appreciated by viewing cleared skins from the outer surface with the low power binocular microscope. The main supply vessels rising from the "subcutaneous plexus" are also larger in active zones

The large guard hairs of the groups form club hairs and ascend to the surface considerably before their surrounding "fur" fibers. This order of events is just the opposite of that in rat skin, where the larger follicles are delayed in club-hair formation, particularly the monotrichs As larger follicles in the rabbit generally contain vascularized papillae, there is, in this animal, an apparent association between early club formation and the possession of blood vessels in the papilla. Therefore, we conclude that within a hair group there is no widely applying rule connecting the possession of a vascularized papilla with more persistent growth

IV The Vascular Plexuses

A *Idealized Plan*

Quite a number of mammalian skins have been examined by various workers and the existence of well-defined blood plexuses is established It is perhaps valuable to review the subject and to see what possisble relations there may be to hair growth

We can best describe the variations in different animals by giving an idealized picture of skin with the greatest number of plexuses as in Fig. 14. In the general case the outer half of the dermis shows, first, the fine subepidermal plexus (1) and, just below it, the dermal plexus (2) which is wider meshed and consists of stouter vessels Between the dermis and hypodermis there is the so-called hypodermal plexus (3) If a panniculus carnosus is present there are two plexuses, one on its outer surface (4) and one on its inner surface (5). The lowest plexus (6) occurs in the subcutaneous tissue between skin muscle and body muscle (or bone, cartilage, etc). We shall refer to these plexuses subsequently as Nos. 1, 2, 3, etc

B. *Comparative Review of Cutaneous Plexuses*

1. *Mouse and Rat*

In smaller animals like the mouse and rat there seems to be no separate dermal plexus (2) but an obvious subepidermal plexus is present as the illustrations of previous sections have shown In the rat the possible equivalent of the dermal plexus is the apparent linking together of fine vessels at the level of the sebaceous glands, as illustrated in Figs. 2 and 6. The richest plexus in the rat skin (at the regions studied) is No. 4 lying on the surface of the panniculus carnosus. It differs from

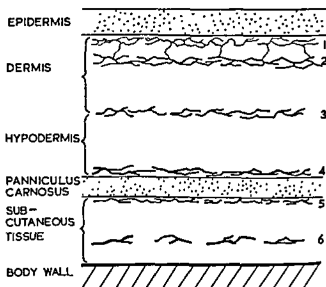


Fig 14 Generalized scheme of blood vascular plexuses of the skin Explanation in text

the others in that the supply vessels and the anastomoses are in the same plane, while in the other skin plexuses the anastomoses are in a [illegible] supply vessels. [illegible]

of wavy vessels (5) beneath the skin muscle We have made no detailed study of a lower fascial plexus such as No 6, but a plexus of this nature is indicated in Fig 6.

Apart from the dermal plexus the other network which seems to be absent in the rat is No. 3 While some fine vessels, such as those within the layer of hypodermal fat, may represent this plexus where there is a panniculus carnosus, in regions where there is no skin muscle a well-

developed hypodermal plexus (3) is present as illustrated by Durward and Rudall (1949, Fig. 9). In the rat, because plexus No. 4 is so well developed and the tissue between skin muscle and dermis is so thin, it seems that a plexus (3) is unnecessary and if present is very poorly developed.

2 Rabbit

In a larger animal, the rabbit, both subepidermal and dermal plexuses are present and in this case the dermal plexus is very conspicuous. As in the rat the great supply plexus is No. 4, illustrated in Fig. 10, but a separate plexus can now be seen between dermis and hypodermis, i.e., No 3. However, this is very feebly developed and consists mainly of fine vessels forming an open meshed net. Below the skin muscle there is a planar plexus (5) while the deepest plexus (6) has not been looked for.

3 Guinea Pig

Among the rodents we have studied the guinea pig which has the additional interest of presenting a pelage which lacks any suggestion of progressive waves of hair growth (Dawson, 1930, Strangeways, 1933) As in the rabbit, plexuses Nos. 1, 2, and 4 are present, but now there is also an extremely well-developed plexus No. 3 It lies above a dense, but not very thick, layer of fat cells which occupies the whole hypodermis between dermis and panniculus carnosus. Large vessels supply it and lie in the one plane and the plexus is made especially conspicuous by very numerous small vessels forming a fine-meshed network in the same plane; they do not penetrate through the underlying layers of fat cells. Below the skin muscle there is another planar plexus (5), while no search has been made for a deeper plexus (6)

4. Dog

The principal observations on other animals have been made by Spalteholz in the dog (1893) Goodall and Yang in the ox (1954), and Ryder in the sheep (1955a, b) It is not easy to relate observations on the dog by Spalteholz to those of the scheme in Fig. 14. He does, however, describe a dermal plexus (2) at the mid-level of the corium, while the hypodermal plexus consists of extraordinarily long vessels which are the equivalent of those running above the panniculus in the rabbit (Fig. 10). They may be regarded as the main supply vessels to the skin

5 Ox and Sheep

In animals like the ox and the sheep the blood supply of the skin is readily described in terms of Fig 14. Plexuses which are subepi-

dermal (1) and dermal (2) are easily recognized in the descriptions given by Goodall and Yang (1954) for the ox fetus and 3-month-old calves, and by Ryder for the fetus, lamb, and sheep of many breeds. Both these animals have a well-developed hypodermal plexus (3). Ryder casts some doubts as to whether plexus No. 2 is always present, in the advanced fetus which we have studied, the situation is very similar to that illustrated by Goodall and Yang for Ayrshire embryo and calf. Ryder establishes the point that in sheep the main supply to the skin is by a fascial plexus of large vessels lying beneath the panniculus carnosus. In an examination of our advanced fetus we see this plexus clearly but also all the plexuses of Fig. 14 In this material there is no dermal or hypodermal fat, but much subcutaneous fat beneath the skin muscle There is a well-defined region of loose fascia constituting the hypodermis between dermis and panniculus carnosus. A hypodermal plexus is present at the level (3) while much less conspicuous plexuses are present above and below the panniculus carnosus (4 and 5).

In the sheep fetus many finer vessels are distributed throughout the lower half of the dermis, and although lying mainly horizontally, they form three-dimensional networks As a very similar situation is suggested by Goodall and Yang's Plate I, Fig 3, for the skin of a 3-month-old calf, it would seem reasonable to associate these vessels with the growth of the "pars fasciculi" of the dermis, as we have defined it in the introduction Possibly an additional plexus of considerable dimensions may form from these vessels, especially where a substantial layer of dermal fat develops as at certain regions in sheep (Frolich *et al*, 1929).

6 Man

Finally, in man, plexuses Nos 1, 2, 3, 4, and 5 are recognized in the scalp, while in the main body regions only Nos. 1, 2, 3, and 6 are present Here, of course, we are equating the vessels of the dermal papilla with a subepidermal plexus

The only plexuses which seem closely connected with hair growth are Nos 1, 2, and 3, or if No 3 is absent, then No. 4 is important Parts of the follicle system are supplied from the above plexuses or from branches between them. Our review shows that there are satisfactory terms for plexuses Nos 1, 2, 3, and 6, namely, subepidermal, dermal, hypodermal, and fascial At present, however, we are not able to suggest a suitable term for the plexus lying on the surface of the panniculus carnosus, for it varies from being a relatively insignificant supply of localized importance to being the main supply network to the skin as in the rabbit and rat It appears that at the positions 3, 4, and 5, there

is always some kind of plexus; these levels are in the nature of interfaces between different tissue structures

C. *Vascular Plexuses and Hair Growth*

We see no significant relation between the main plan of the plexuses and the nature of the hair growth In a less comprehensive view one might think there is some relation, for in the mouse, rat, and rabbit, where hair growth waves are so characteristic, there is absence or very poor development of a hypodermal plexus, while in the guinea pig where there are no growth waves, this plexus is very well developed. But in the rat, in body regions lacking skin muscle, a hypodermal plexus is well developed and hair waves are still quite evident. The only relation we have observed is that, at regions of active hair growth, supply vessels in the dermis may be of greater caliber than in quiescent zones.

Much remains to be learned about these blood supplies; in particular we need to perform separate injections of arterial and venous systems and to explore the relations of each of these to the follicle system.

V. Summary

Follicle development and hair growth can take place in the absence of any blood circulation as in tissue culture. However, *in vivo* a blood supply is necessary for maintenance of active hair growth, bringing nutrients and oxygen as well as various hormones There are two principal regions of the follicle which are supplied, namely the hair papilla and the outer surface of the lower half of the follicle

In the smallest hairs of the rat and rabbit there are no blood vessels in the papilla and, indeed, within these small organs a blood corpuscle would be a clumsy object, but in the largest hairs a papilla circulation is present Well-developed papilla vessels are known to occur in man, ox, sheep, guinea pig, rabbit, and rat, *but are perhaps absent in the* mouse. In most of these cases we can say that if the papilla reaches a certain size it will have blood vessels within it. We might suspect that the sheep shows the smallest follicles which have a well-developed papilla circulation A feature of sheep papillae is that they contain far more densely packed cells than the papillae of man, rabbit, and rat A possible view would be as follows: if the papilla is large, or being small it has a denser structure, then the flow of metabolites is insufficient in the absence of a special blood circulation.

The vascular system of the follicle wall consists of a network of capillaries lying just outside the circular layer of connective tissue fibers which cover the glassy membrane and the outer root sheath Extraordinary variation occurs in the extent of this system of vessels. In the

smallest hairs of the rat there is no closely investing network, but instead a number of vessels runs fairly near the follicles in the tisssue between them. In the largest follicles very dense networks exist and these extend over the whole lower half of the follicle length. We have seen nothing like this density of capillaries in the rabbit or guinea pig, but Unna (1908) refers to some very closely meshed networks in his material from the human fetus and newborn In spite of the circular layer of fibers and the glassy membrane it is reasonable to assume that the network of vessels is, in some measure, concerned with activity in the outer root sheath. Two contrasting follicle wall patterns are as follows. (a) a close network of capillaries completely investing the follicle below the level of keratinization, (b) a network largely restricted between the level of keratinization and the upper level of the follicle bulb, the bulb surface itself being comparatively free of vessels. The former type is characteristic of monotrich and awl follicles of the rat, where at least in the awl there are no blood vessels in the papilla. The latter type is characteristic of sheep and calf. There is the suggestion that the dense vessels on the follicle bulb may reinforce or serve instead of *a papilla circulation, while those at and above the follicle neck* have another purpose, namely, they are concerned with activity in the outer root sheath.

In the general plan, the blood plexuses of the skin are comparatively uniform in many animals. There is evidence that in any substantial area which is growing hair, the vessels in the dermis are enlarged, for example, in the active zone of a growth wave or as a result of epilation by pulling This may cause the spreading of growth into an adjacent quiescent area, since parts of this are inevitably connected via a plexus with the dilated vessels in the active zone Related to this is the evidence, that the earlier start of a new growth phase in monotrich and awl follicles of the rat is due to the greater vascularization of resting follicles of these types

In animals which show these regular waves of hair growth, the length of the growing period of neighboring fibers is more or less the same. This uniformity of growing period is not found in the guinea pig and this animal shows no hair growth waves We have made some study of the vascular system in guinea pigs and do not find a grouping of blood vessels but rather the circulation to each follicle is separate It may be generally true that where there is synchronous growth within a region the vessels of large groups of follicles are intimately connected together It is possible that the growth periods might be less uniform if the follicles were entirely separate In animals such as rabbit, rat, and mouse, it is important to know how an isolated fiber behaves We

attempted to study one aspect of this problem by pulling out single guard hairs in the rabbit, but the procedure failed to induce a regrowth which suggests that pulling, in itself, does not provide the necessary stimulus. Where other areas had all hairs pulled the regrowth of all types occurred normally.

Another aspect of the vascular supply to follicle groups concerns the intercommunications between them, some of which we have illustrated These circuits will be difficult to work out, but knowledge of them may be rewarding. A striking feature is the persistence of so many vessels during the resting stage. These are of small caliber but appear to be quite open as dyestuff injected into the living animal flows readily in these vessels. This rich supply to a so-called resting follicle suggests that there may be considerable metabolic activity there.

VI. References

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CHAPTER 10

The Vascularity and Innervation of Human Hair Follicles

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I. THE BLOOD VESSELS

The capillary networks that surround the hair follicles of several species of animals have been described in Chapter 9 of this book, and those of the follicles of wool fibers have been demonstrated by Ryder (1956a). For these demonstrations blood vessels were injected with India ink. There is always a question, however, in such procedures, as to whether all of the capillaries have been injected Montagna and Ellis (1957b), and independently Klingmüller (1957), have shown that the endothelium of capillaries and some end-arterioles react specifically for alkaline phosphatase By applying the azo-dye techniques for alkaline phosphatase (Gomori, 1952) to thick frozen sections of skin fixed in formalin, the vascular beds around the hair follicles can be demonstrated clearly, as shown in the illustrations which follow, naturally, these illustrations have been chosen for their photogenicity. The details of this beautiful and useful technique may be found in the paper by Montagna and Ellis (1957b)

Hair follicles are surrounded by a dense and continuous plexus of capillaries, the pattern of which is different in active and in quiescent follicles (*Figs. 1 and 2*)

The lower third of active follicles, from the base to just above the bulb, is enveloped by a rich vascular plexus composed of long, more or less parallel vessels connected by cross-shunts (Figs 3 and 4). The parallel vessels arise from the hypodermal plexus, and course straight down to the papilla pore (Fig 3); the horizontally oriented cross-shunts are often tortuous and have secondary shunts that outline a latticework pattern (Figs 1, 3, 4) The bore of the longitudinal vessels is larger than that of the cross-shunts, and for this reason they are assumed to be terminal arterioles From just above the bulb, up to the level of the sebaceous glands, most of the cross-shunts drop out, and the follicle is surrounded only by a palisade of parallel vessels. In thick preparations these can be traced from their emergence at the papilla pore all



FIG 1 Montage of the lower two-thirds of an active hair follicle in the scalp. The lower capillary plexus at the keratogenous zone, and the parallel vessels are evident Magnification × 55

FIG 2 Montage of a quiescent hair follicle from the scrotum showing the dense bundle of vessels at the base of the dermal papilla Magnification × 55

the way to the level of the sebaceous glands, where once again the vessels form a network that envelops both the glands and the pilary canal (Fig. 5). The parallel vessels lose their identity around the pilary canal and terminate in a loosely woven network that extends to the surface of the skin. Loops of capillaries form a vascular ring around the orifice of the follicle under the epidermis, this collar is continuous with both the vascular loops of the epidermis and the network around the follicle Sebaceous glands are richly vascularized by baskets of capillaries that surround the acini and follow all of their contours.

The dermal papilla at the base of the follicles contains a large tuft of capillaries (Fig. 6), which extends to the walls of the inner side of the follicle and are often practically in contact with the wall. The capillary tuft of the papilla arises from the longitudinal vessels that stem from the hypodermal plexus, makes a straight descent down the sides of the follicle, flows around the lip of the papilla pore, and enters the dermal papilla from below. The vascular system of each follicle is a continuous unit from the dermal papilla to the area around the infundibulum, including the plexus around the sebaceous glands.

The follicles of vellus hairs in the body skin or in the bald scalp are surrounded by very simple capillary systems. A few capillaries surround the lower part of the follicle, but no vessels penetrate the dermal papilla, which is much more reactive for alkaline phosphatase than that of large follicles (see Chapter 20, p 480 of this book). The enormous sebaceous glands associated with these follicles are highly vascularized There are many follicles intermediate in size between those of vellus hairs and those of small overhairs, and the vascularity of their dermal papillae show a graded transition from one type to the other There is also an inverse relation between the amount of phosphatase activity and the fullness of the capillary plexus

During the transition from the active state to the quiescent one, the bulb atrophies, and the base of the follicle moves upward in the dermis and partially out of the network that surrounds it The dermal papilla also recedes upward and slips away from its central capillary tufts Most of these capillary nets remain intact, but are collapsed at the base of the resting follicle as a tangled bundle below and around the free dermal papilla (Fig 2) Since only the lower part of a follicle degenerates when it becomes quiescent, the palisade of parallel vessels in the middle third remains unchanged Higher up, around the sebaceous gland and the infundibulum, the plexus also remains intact When a quiescent follicle becomes active again, the new bulb plows its way through the bundle of capillaries and grows inside it.

The capillary networks from any part of a follicle and its sebaceous

glands are a continuous system, and each vascular bed is a unit. Since the pilosebaceous system functions as a unit, the vascular mechanism may be responsible for this integration or at least shares the responsibility.

Nearly the entire lower third of the follicle consists of the bulb, in its lower end, the cells of the matrix proliferate constantly, move up, and form the hair and the inner root sheath. In its upper part, the cells that have come from the matrix grow many times their original volume and synthesize keratin; the final phases of keratin formation take place in the keratogenous zone in the upper limit of the bulb. At this level of the follicle, the cells of the outer root sheath contain more glycogen than those in other levels (Montagna, 1956; Montagna *et al*, 1951) and more esterases and β-glucuronidase (Montagna, 1955, 1957), it is also surrounded at this level by a glassy membrane that is thicker than at any other level These characteristics suggest that this region of the outer root sheath is a very active tissue. Ryder (1956b) has given the strongest evidence for this by showing in the follicle of sheep that only 6 minutes after the injection of cystine labeled with S^{35}, a strong accumulation of radioactive particles can be recovered first around the general region of the keratogenous zone. This is the region of the follicle surrounded by the densest capillary networks in the sheep, the rat (Durward and Rudall, 1949), and man. The region of the upper part of the bulb is unquestionably an important site of exchange.

Exchange must also take place through the dermal papilla The amount of vascular tissue in a dermal papilla is dependent on its size. the wider it is, the larger the capillary tufts it contains (Ryder, 1956a). The dermal papillae of active human follicles are very wide and contain large quantities of endothelium. During periods of quiescence, either the tissue of the papilla disengages itself from the capillary tuft by flowing away from it or the capillaries atrophy. Such large numbers of capillaries in the dermal papilla strengthen the belief (Montagna, 1956)

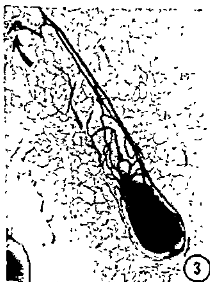
FIG 3 Blood vessels around the lower part of an active hair follicle from the scalp The parallel vessels originate from an artery of the hypodermal plexus which is shown in cross section (arrow) Magnification × 45

FIG 4 Two large active hair follicles from the scalp showing the rich capillary plexus around the keratogenous zone, as well as the parallel vessels and the capillary tufts in the dermal papillae Magnification × 55

FIG 5 The capillary plexus around the upper end of a follicle in the scalp Magnification × 90

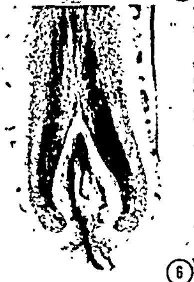
FIG 6 A sagittal section through the bulb of an active follicle in the scalp showing the capillary tuft in the dermal papilla

that the bulk of the papilla cells remain relatively the same during periods of follicle activity and quiescence and that the changes in the size of the papilla are largely due to the increase and decrease in the amount of capillary tissue and to the increase and decrease in the size of the papilla cells.









II. The Nerves

Stiff hairs, called vibrissae, grow around the muzzle and the face of all mammals except man. These are sensory hairs and their follicles, which are surrounded by a characteristic blood sinus, are enwrapped by numerous sensory nerves and end organs (Vincent, 1913; Melaragno and Montagna, 1953). Vibrissae, however, are not the only sensory hairs on the mammalian body. The follicles of many of the hairs of the face, and those in the perianal and genital regions also have different amounts of innervation The follicles of hairs elsewhere on the animal's body are rarely associated with nerves. Although a thorough regional survey has not been made of the hair follicles of the human body, more of them are innervated than in other animals investigated, and they may apparently be found anywhere on the body. The brief descriptions that follow are based on the follicles of the scalp, although generalizations can be made, let it be clearly understood that the patterns of innervation are not the same in all hair follicles and that many follicles have no nerves at all.

Hair follicles are surrounded by a network of interlocking nerve fibers that converge toward two or more nerves from the cutaneous plexus. These run a straight course along the sides of the follicle from the deeper cutaneous plexus (Weddell, 1941, 1945, Wollard *et al*, 1940). Only the upper part of the follicle, just below the entrance of the sebaceous glands, is surrounded by nerves, the lower half or two-thirds is always free of them (Fig. 7). If nerves are there, as figured in most existing diagrams, we have never succeeded in demonstrating them with any of the techniques employed. Below the level of the sebaceous glands, the nerves form a collar that is denser in its upper than in its lower limits (Fig 8) From this plexus fibers extend to the upper part of the pilary canal and form a sparser network around the infundibulum of the follicle (Fig. 9), a few scattered fibers bear a casual relationship to the sebaceous glands Very thin fibers from the follicle plexus rise to the base of the epidermis, divide into barely visible branches, and penetrate the epidermis Fibers from the follicle network also join the nerves to the arrectores pilorum muscles and those around the eccrine sweat glands. All of the nerves described here contain specific cholinesterase, demonstrable with acetylthiocholine iodide (Montagna and Ellis, 1957a) These nerves are so richly supplied with this enzyme that histochemical preparations give an excellent demonstration of their distribution (Figs 7-10). When hair follicles become quiescent, they become shorter, and the nerve collar around the stable part of the follicle is closer to the base than it is in active ones Quiescent follicles may become so short that their base partially slips out of the



FIG 7 Nerves in the scalp demonstrated by the cholinesterase technique The nerve plexus around the hair follicle in the middle of the field (bracket) is seen in greater detail in Fig 8 Magnification × 20

FIG 8 The entire, larger nerve plexus around the follicle at the level of the sebaceous gland The upper part of the plexus is much more dense than the lower part Magnification × 90

FIG. 9 The arrows indicate the upper, sparser nerve plexus which is very delicate and located just beneath the epidermis Magnification. × 90

FIG 10 The partially collapsed nerve plexus around the base of a quiescent hair follicle Magnification × 160.

nerve plexus, which collapses or shrinks at their base (Fig. 10). The nerves, however, remain intact and rich in cholinesterase.

That these nerves contain specific cholinesterase, is indicated by their reactivity with acetylthiocholine iodide and not with butyrylthiocholine iodide. The complete inhibition of the reaction by physostigmine is final proof of this specificity.

Specific cholinesterase, then, can be demonstrated in motor as well as in sensory nerves. Since the nerves around them subserve touch, hair follicles must be tactile sense organs (Weddell, 1941, 1945). The function of hair follicles surrounded by nerves is similar to that of the follicles of the vibrissae (Vincent, 1913), and in both cases, the nerves are reactive for specific cholinesterase (Montagna and Beckett, 1958). Cholinesterases have also been found in Meissner and Pacinian corpuscles in the human finger and in the nerves that emerge from them (Beckett *et al*, 1956) The free nerve endings in the epidermis, which subserve pain (Weddell, 1945), also contain appreciable cholinesterase. This is not easily reconcilable with the concept that the presence of cholinesterase is rare outside of the parasympathetic nervous system

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CHAPTER 11

The Behavior of Pigment Cells and Epithelial Cells in the Hair Follicle[1]

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I. INTRODUCTION

The general mechanism of growth of the hair is known (Oyama, 1904; Dry, 1926, Butcher, 1934, Schwanitz, 1938, Chase *et al.*, 1951, Auber, 1952, Chase, 1954) but certain additional details concerning the behavior of epithelial cells and pigment cells during the hair cycle may be relevant to a more complete understanding of the processes of hair growth.

II. DEVELOPMENT OF EPITHELIAL CELLS

There are two centers of intussusceptive growth in the early anagen or "embryonic" stages. One is the upper, permanent external sheath including part of the capsule around the club of quiescent follicles, and the other is the cell group attached to and capping the dermal papilla. From the first is formed the lower external sheath, from the second is formed the elongated bulb that surrounds the dermal papilla The lower portion of the elongated bulb becomes the true matrix, the ring of rapidly dividing cells in the later anagen or hair-producing phases of the cycle The cells of the cap also form the first keratinized product of the new growth, the upper part of the inner sheath (Chase *et al*, 1951)

The matrix cells form all of the lower and subsequent inner sheath This early internal sheath does not push up through the solid external sheath for any appreciable distance, but the sheath remains relatively fixed in position and is added to on the proximal end as the bulb

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elongates and the whole follicle grows in length. Only when the follicle ceases its elongation do the inner sheath products of the matrix ring push the distal sheath outward (Fig 1). By this time the hair is being formed and will push through the end of the inner sheath, then through the cell mass immediately below the sebaceous duct, and thus into the pilosebaceous canal. The cavity under the early inner sheath, which is soon filled by the hair, is the result of the descent of the bulb



Fig 1. Unstained section Comparatively short follicle on right just starting to produce a hair Note club and capsule of previous hair generation On left is inner sheath enclosing new hair, the old club has been mechanically dislodged Magnification × 300

In the wall of the follicle, mitotic activity ceases in the lower external sheath with the accumulation of glycogen Around the papilla most mitoses of the matrix are lateral, i e, perpendicular to the papilla, and give rise to the three concentric columns of the inner sheath and to the cuticle and cortex of the hair. Bounded by the cylinder of the cortical cells, the medullary cells become arranged to fill the space. In the small zigzag hair of the mouse a single column arises from a small ring of these medullary cells A new cell passes into this canal about every 10 minutes as if they were passing from an outer, flared part into the tube of the funnel (Fig. 2). The possibility that the medullary cells are the result of intussusceptive growth in the matrix and are thus slip-



Fig 2 Unstained section A follicle to show how medullary cells are "funnelled" in a column Magnification × 300.

ping off beyond the end of the pointed papilla to fill the space within the cortical cylinder is an attractive hypothesis but seemingly is contraindicated by the present evidence Instead, the distal portion around the dermal papilla appears to be relatively stable, it is the structural framework in which the melanocytes are embedded, and it is the central "stop" around which the cells for the inner sheath and hair must pass and then converge.

Although cells from the matrix may be seen to be in rows or streams, such that the most proximal one gives rise to *Henle's* layer, the next to Huxley's layer, etc (Chase *et al.*, 1951, Auber, 1952), this may be largely the result of a fortuitous angle of sectioning The mass of cells from the matrix ring seems to be comparatively indeterminate A manipulation of the very turgid bulb can sometimes rearrange the "loose" cells and a normal hair will develop following a "fault" in the shaft. As the cells link or cement in keratinization they can no longer be exchanged.

The differentiation into five concentric columns plus the medulla are probably the result of different pressures (Auber, 1952), involving the rigid Henle layer and cortex and the incompressible, turgid Huxley layer and medulla. It appears possible that the cortex determines the size of the outer cylinders; but when an irregularity occurs in the cortex due to an unusually large pigment clump or whole pigment cell becoming incorporated into the medulla, this bulge is not reflected in the Henle layer. At present, in the mouse at least, it appears that the Henle layer is the basic funnel determining the sizes of the other cylinders In a zigzag follicle when a constriction is being formed (Fig. 3), usually with no medullary cells or with a few arranged longitudinally rather than transversely, there is a smaller mass from the matrix cells and the cylinders are all smaller in diameter.

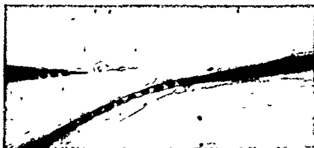


FIG 3 A zigzag hair showing constriction in which medullary cells are arranged longitudinally. Magnification × 300

III. Behavior of Pigment Cells

Pigment cells are located in the upper external sheath adjacent to and often protruding into the dermis or connective tissue sheath, just as they are in the basal layer of the epidermis. In the bulb they are correspondingly in contact with the dermis of the bulb, the dermal papilla In the elongation of the follicle an occasional pigment cell may be found which has been carried along by epithelial growth from the upper, permanent external sheath Likewise, an occasional one may be observed temporarily in the elongated portion of the bulb, the matrix, carried from the original upper bulb Such a stray melanocyte is clearly the reason for the rare, early appearance of pigment in the inner sheath. Such a cell is soon depleted of its pigment granules and also soon loses its attachment to the basal layer and may even become incorporated into Huxley's layer.

Such a distortion and abnormal location of a melanocyte can be produced experimentally. When a turgid bulb is pressed in a distal to

proximal direction with a needle, cells can be so displaced that two or three pigment cells will have descended to the lower bulb. If such a follicle remains competent, there will be pigment granules and melanocyte remnants in the inner sheath during the next day's growth

When pigment cells normally first develop melanin in anagen III (Chase *et al.*, 1951), their long dendrites follow the lines of growth in the bulb Soon they remain confined to the upper bulb region, become relatively large, the karyocytes project somewhat into the dermal papilla, and the dendrites become shorter. By a device not yet clear they inoculate granules into the passing stream of cortical and medullary cells. In some cases the dendrites appear to be torn off and remain on the distal sides of the passing cells The granules in some cases remain between the cells, at least for some medullary cells, but most of them are engulfed apparently into the recipient cells. In dilute mice of the genotype *dd* the melanocytes have clumped pigment, the clumps being torn off and generally incorporated between the cells rather than within them In this case the whole melanocyte is also frequently dragged into the stream of cortical and medullary cells. In the Light mouse, $B^{lt}B^{lt}$ (Chase and Quevedo, 1955, Quevedo, 1956, Quevedo and Chase, 1956), most of the mature melanocytes are lost into the stream leaving no mature ones for the latter part of the hair growth. In albino mice, large amelanotic melanocytes (Taylor, 1949, Chase *et al.*, 1951, Silvers, 1953, Quevedo, 1956) project into the dermal papilla and apparently deliver amelanotic particles to recipient cells These melanocytes are completely normal except for lack of the enzyme to form melanin. They are even injured by x-irradiation (Quevedo, 1956) in the same manner as normal melanocytes. A white hair induced by x-irradiation of a colored animal has no mature melanocytes in the bulb, the upper bulb having cells of a uniform size with none noticeably larger or projecting into the cavity of the dermal papilla. This bulb resembles in every detail the bulb of a white area of a white-spotted animal (Chase and Rauch, 1950) and is unlike the nonirradiated albino bulb The follicle of a Light mouse, when producing the white base of the hair, has a bulb which also resembles the white-spotted or irradiated bulb, and not the normal albino bulb

The density of pigmentation in the upper bulb in normally pigmented follicles obscures the details of the behavior of pigmentary and recipient epithelial cells (Fig. 1). Pink-eyed dilute, *pp*, mice and x-irradiated mice in which some follicles produce only one or two mature pigment cells are useful for these studies (Chase, 1949, Chase and Smith, 1950). From follicles of this latter sort mosaic hairs arise. A single melanocyte "feeds" some recipient cells, others receive only a few

granules, and still others receive none. If the melanocyte is near the tip of the upper bulb, it may contribute to all of the cortical cells of the tip of the hair and to the medulla cells, but further proximal and especially in the larger hairs it is evident that many hair cells escape being fed pigment. In the larger, normally pigmented follicles, frequently hair starts to form before the pigment cells are ready to inoculate, resulting in unpigmented tips. The "life" span of a mature melanocyte is long, approaching the length of the growing time of the hair in the mouse. There may be replacements but they are rare and have never been detected in the mosaic cases If mitosis does occur in a pigmented melanocyte, it occurs in the early stages and not in the short-dendrite, mature form More mitosis of melanocytes may occur in sheep and in man In normally pigmented mouse hairs, there is seldom an "uprooting" of a melanocyte, but as the end of hair growth approaches, the melanocytes regress in size, produce fewer new granules, and often the granules remaining are liberated into the dermal papilla The inhibition of melanogenesis occurs before the complete inhibition of growth (Chase, 1954, 1955).

What is the source of melanocytes for the succeeding hair generation? They may occur by revivals of the former melanocytes which have become dormant, they may arise from stem cells in the epithelium, which are themselves unpigmented, or they may arise from cells in the dermal papilla as suggested for feathers (Foulks, 1943). By observing successive mosaic hairs from the same follicle, it is apparent that although not identical in pattern, there is a similarity in amount of pigment (the same or less, never more) and in the general arrangement. In $B^{u}B^{u}$ mice where some melanocytes are clearly uprooted, the next generation has an apparently new set of melanocytes; although later hairs have somewhat less pigment, there is not the loss which would be expected from the observed incorporation of mature melanocytes into the hairs. The possibility of a dermal source has yet to be conclusively proved or disproved The bulk of evidence supports the contention that there are stem cells at definite sites which give rise to the mature and expendable melanocytes, these cells enter the epidermis from the neural crest (Rawles, 1940) and become embedded in the basal layer. When the daughter of a stem cell (or the stem cell itself) becomes melanogenic, there is a tendency for it to protrude into the dermis, from the epidermis or from the upper bulb, which is a displaced portion of the epidermis. The melanocytes, and presumably their ancestral stem cells, are spaced and have definite sites, as is particularly evident in follicles producing mosaic hairs where most of the sites have been inactivated or destroyed by x-irradiation

Catagen is the short transition stage between active anagen growth and the resting telogen stage. It is the time of drastic shortening of the follicle. Relatively little is known about the mechanics of this stage. The turgidity of the follicle, as explored by micromanipulation, decreases rapidly and the dermal papilla cavity opens to leave a rounded, condensed dermal papilla. The club, which is the anchoring end of the resting hair, forms at this time from cells that would have formed cortex, cuticle of the hair, cuticle of the inner sheath, Huxley's layer, and Henle's layer. The formation of medulla stops shortly before this time (Dry, 1926, Chase, 1954), and thus the medulla takes no part in formation of the club (Fig. 1). This process of club formation does not involve the upper bulb: in fact it does not occur while the upper bulb is still in its normal relative position. The club and its surrounding epithelial capsule form from matrix cell descendants, the more keratinized cells being the club filaments, the unkeratinized cells to which they are attached being the capsule. The upper bulb, now flattened, but still attached to the dermal papilla, follows the club, but is separated by the large mass of normally degenerating cells. These degenerating cells are mainly those of the lower external sheath which were earlier laden with glycogen.

If the upper bulb is normally the main source of what has been called the "germ" of the telogen, then the sites of stem-cell melanocytes should be detectable. A few pigment granules are often visible in unstained material, but they appear to be pigment remnants from the preceding generation (Fig. 4). Occasionally, in an anagen II stage, a precocious melanocyte is observed in unstained material. Except for the pigment it appears similar to the other cells which border the resting dermal papilla.

Particularly illuminating in this connection is the type of catagen-telogen and subsequent anagen which occurs following x-irradiation of a growing follicle (Chase and Rauch, 1950, on the mouse; Montagna and Chase, 1956, on human scalp follicles). Here degeneration is more extensive and no club and capsule are formed. The anagen is delayed but when it occurs, growth is almost entirely from the upper external sheath, a true regeneration. Various anomalous situations may be due to the loss of the normal upper bulb "germ" and its regeneration from the upper external sheath with the potential melanocytes which are not usually called upon to produce pigment granules for the hair. The following situations may be explained in this way: the loss of pigmenta-

creases in pigmentation in man following irradiation The upper sheath, continuous with the basal layer of the epidermis, constitutes a reserve supply of epithelial cells and potential melanocytes, which, unless completely inactivated, can respond to a dermal papilla

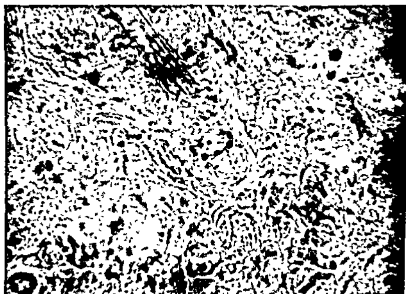


FIG 4 Unstained section A resting follicle showing a club with capsule, a rounded dermal papilla, and a "germ" which contains some pigment clumps from previous hair growth. Magnification × 600.

At present, there is growing evidence that the lower part of the external sheath forms from cells of the upper external sheath, of the capsule, and that the lower bulb (the matrix) forms from the "germ" which remains relatively stable as the upper bulb and definitive site of melanocytes.

IV. ACKNOWLEDGMENTS

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CHAPTER 12

The Electron Microscopy of Human Melanocytes and Melanin Granules

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I. INTRODUCTION

Integumentary melanin is produced only by the melanocytes. The many accounts of the formation of melanin, based on observations with the light microscope, contain extremely diverse views on the particular cell constituents involved in this process. Some authors (Meirowsky, 1908, Meirowsky and Freeman, 1951) maintain that melanin granules arise from extruded nuclear material, others (Rényi, 1924, Makarov, 1929, Woods *et al*, 1950) regard mitochondria as the precursors, while others (Guttes, 1953) favor the Golgi apparatus as the site of their formation. Most of these studies were made on the embryonic pigment epithelium of the retina or on melanotic tumor cells, there is little information about the detailed cytology of the dendritic melanocytes of the integument The histology and function of these cells, however, have been ably dealt with (Masson, 1948, Billingham, 1948, Billingham and Medawar, 1953, Medawar, 1953). In histological preparations the pale cytoplasm of melanocytes is usually shrunken and unfavorable for fine cytological observation, most observations have been concerned with their general form, location, and numbers as revealed after coloration with methylene blue, the dopa reaction or gold impregnation. Although the details of melanogenesis may vary in different tissues and

The electron microscope with its greatly extended range of resolution offers a promising new line of attack. A great variety of tissues has already been examined with this instrument and the structural charac-

teristics of the mitochondria, Golgi apparatus, and endoplasmic reticulum are sufficiently well established to enable one to identify them with confidence in well-prepared material

The observations which follow have been made mainly on human hair follicles, this is by far the easiest material in which to study melanogenesis in man Hair follicles can be obtained in quantity by plucking scalp hairs and selecting for fixation those follicles with well-developed bulbs The wide range of human hair color also affords the opportunity to study the relationship between color and the size, structure, and development of the pigment granules

Hair follicles secured in this way usually include only the upper portion of the bulb, but occasionally the lower part, together with the papilla, is also present Although some cells are no doubt injured by this method, sufficient numbers of them are found with presumably undamaged fine structure Earlier studies (Barnicot *et al.*, 1955), made on material embedded and sectioned in methacrylate polymer, after fixation in Palade's buffered osmic acid solution and examined under a Philip's electron microscope, did not yield as detailed information as the later studies of material embedded in epoxide (Araldite) and viewed with the Siemens Elmikroskop I.

Suspensions of isolated melanin granules have also been examined Melanin was isolated from hair by a variety of methods, including boiling in *N* KOH, prolonged hydrolysis in 3 *N* HCl, extraction with hot phenolthioglycolic acid mixture (Laxer *et al.*, 1954), or digestion with papain in the presence of sodium metabisulfite and urea (Lennox, 1952)

II. The Structure of Isolated Melanin Granules

Isolated melanin granules are examined as dried suspensions to study accurately their differences in size and shape Other authors, studying material from various sources, but prepared in this way (Mason *et al*, 1947, Shackleford, 1948, Laxer *et al*, 1954, Lion *et al*, 1957), have shown variations in the size and shape of the granules, depending on the species or tissue from which the material was obtained. Under the electron microscope the granules from dark human hair appear as dense, ovoid or rod-shaped bodies, the length of which varies from about 0 4 to 1 0 μ and the breadth from about 0.1 to 0 5 μ (Barnicot *et al*, 1955). Although the majority of the granules are about three times as long as they are broad, there is a continuous range from more slender rods to almost circular forms. In transverse cuts of the more mature regions of the hair shaft, the granules, which are aligned with their long axes parallel with the keratin fibers, are circular in cross section. In suspensions of granules, little evidence of internal struc-

ture can be discerned Some of the elongated granules, however, show a streak of lower density down their long axis, some of the more rounded forms may have a less dense central area or a ragged, incomplete margin on one side. These features can be seen even after digestion with papain, which is mild enough to leave some intact residues of keratin fibers.

The internal structure of the granules and the effects of chemical digestion can be studied more accurately by centrifuging the pigment suspensions and sectioning fragments of the sediment (Birbeck *et al.*, 1956). The granules digested with papain have a solid, almost homogeneous interior, but the granules that have been isolated by boiling the hair for 15 minutes at 100°C in *N* KOH show a more open granular interior; sometimes they are reduced to a shell of dense material Refluxing for 24 to 48 hours in 3 *N* HCl damages the granules less than the brief treatment with alkali

The assertion of earlier investigators that the granules in dark hairs or feathers are larger than those in lighter colored ones (Hausman, 1928–1929, Russell, 1949, Shackleford, 1948, Hutt, 1953) is confirmed by the studies with the electron microscope. The granules from Negro hair are conspicuously large, and those from dark European hair, in turn, average larger than those from blond and red hair.

III. The Cytology of the Melanocyte and Melanogenesis

In sections of follicles of pigmented hairs the melanocytes are easily distinguished under the electron microscope since they have numerous, dense melanin granules in the cell body and in the dendritic processes (Fig 1). Portions of dendrites cut in various planes lie freely in the intercellular spaces between developing cortical cells in a large area of the bulb region The connection of these dendrites with the perikaryon can sometimes be found. Melanin granules, which for the most part do not differ appreciably in size or structure from those that have been isolated from mature hair, are widely distributed apparently at random in the cytoplasm of the melanocyte. In thin sections of osmic-fixed tissue, the granules are seen as solid structures composed of a dense, relatively homogeneous material. The fact that they often shatter or break out of the section is evidence of their hardness. Granules with a hollow center are occasionally seen Most of the pigmented granules show a thin shell of a dense, finely particulate material, some, however, have none (Fig 2) This precipitate may perhaps be caused by a local reduction of the osmic acid used in fixation

Other workers have described the processes of epidermal melanocytes terminating in expanded ends on the epithelial cells (Billing-

ham, 1948); presumably melanin is transferred to the epithelial cells at these points. We have not been entirely successful in elucidating the details of the process of transfer. Compact bundles of melanin granules similar in diameter to melanocyte processes are occasionally found lying



FIG. 1. Low-power view of melanocytes and immature cortical cells in the upper bulb region of a pigmented follicle. Two melanocytes (M) and dendritic processes (Pr) lying between cortical cells. *Note wide spaces between melanocytes and cortical cells.* Endoplasmic reticulum (Ret) and part of Golgi region (G) are indicated in the melanocytes. Magnification × 5625.

within the cytoplasm of cortical cells; these seem to be the terminations of melanocyte processes Such aggregates of melanin granules may sometimes be surrounded by a distinct cell membrane. Besides melanin



FIG 2 High magnification field of forming melanin granules in a melanocyte from dark European hair Two granules (M1) showing early stage of melanization of lamella material, left-hand granule (M1) is perhaps a transverse section M2 is a more advanced stage in which the lamella material is thickened, in M3 the lamella structure is barely discernible Note fine particulate material around and inside some of the more mature granules Magnification × 90,000

granules, mitochondria and fragments of reticular material are also found in dendritic processes, these cell constituents are perhaps transferred to epidermal cells, together with pigment granules. The phenomenon of infective spread of pigment-forming capacity, which Billingham and Medawar (1948) have inferred from their studies on grafts between white and black areas in guinea pigs' skin, could be due to transfer of these structures.

Pigmented melanocytes cut in a suitable plane have a differentiated *area distal to the nucleus, which seems to be a site of active melanin* formation. This zone often contains numerous filamentous mitochondria together with many small vesicles (Fig 3). The center of the zone has no melanin granules, but the periphery has small vacuoles about 0 5 μ in diameter, which are surrounded by fine shells or contain convoluted strands of dense material. These are undoubtedly early stages of formation of melanin granules. The granules appear to start as hollow vacuoles in which a tenuous material appears in the form of a folded lamella or sometimes as an incomplete shell near the surface. This material is rapidly thickened and defined by the deposition of more dense material, until a solid body about twice the size of the original vacuole is formed. Some of the granules are elongated even at an early stage and the folded membrane they contain appears in sections as a series of parallel dense strands. In occasional cases these strands have a varicose appearance. Traces of the lamellar structure in more mature granules can be seen in high resolution pictures (Fig. 2). In some preparations a considerable space may exist between the surface of the granules and the vacuole which contains it.

It is generally considered (Mason, 1953, 1955) that the formation of melanin granules involves the deposition of polymerized indole-5,6-quinone on a protein matrix, to which it becomes firmly attached. Perhaps this is brought about by a tanning mechanism similar to that described for the hardening and darkening of some insect cuticles.

Evidence supporting this view was obtained from the examination of follicles from human albino subjects Although the melanocytes of *albinos contain no structure of comparable density to melanin granules,* they can be identified fairly easily by their dendritic process and the highly granular character of their cytoplasm (Barnicot *et al*, 1955). The cytoplasm of both the perikaryon and the processes contains mitochondria and aggregates of double membranes and vacuoles representing the Golgi material and numerous ovoid or rod-shaped bodies 0 5 μ or less in length (Fig 4) These contain strands and lamellae that are comparable to the structures found in pigmented melanin granules, except that they remain tenuous and no deposition of dense material

or further thickening occurs (Fig. 5). This material is presumably a protein matrix on which, owing to the virtual absence of tyrosinase activity in the albino, the dark melanin polymer is never deposited. These granule "ghosts" are conspicuous in the dendritic processes and probably pass in small numbers into cortical cells, although they are difficult to identify with certainty.



FIG 3 View of formative zone of a melanocyte from a human hair follicle. Numerous filamentous mitochondria (Mit) Center of Golgi zone (G) containing numerous small vesicles, peripheral to this area are melanin granules in successive stages of formation (M1, M2, M3) The nuclear membrane (Nm) Magnification · × 21,334

There is no evidence of extrusion of nuclear material or the origin of melanin granules from the nucleus; nothing in these preparations suggests the origin of melanin granules by a transformation of mitochondria. The presence of oxidative enzyme systems characteristic of mitochondria

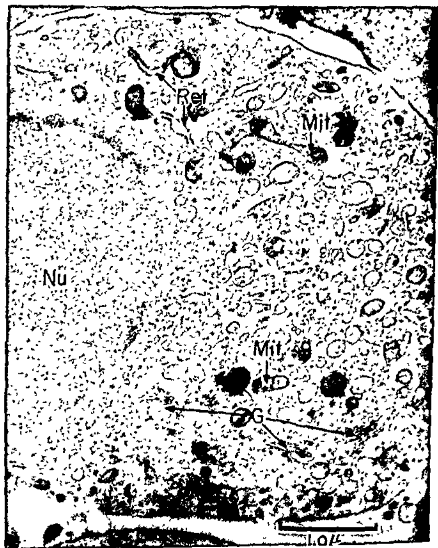


FIG. 4 Low-power view of a melanocyte from a human albino hair follicle The nucleus (Nu), filamentous mitochondia (Mit), endoplasmic reticulum (Ret), [illegible] vesicles (G) [illegible] roducts of the [illegible]

in suspensions of pigment granules from mouse melanoma tissue may mean that mitochondria fragments are present in the suspension (Woods *et al.*, 1950). Under the electron microscope the pigment granules in the cells of Harding-Passey melanomas are spherical bodies containing dense subparticles concentrated near their centers. Mito-

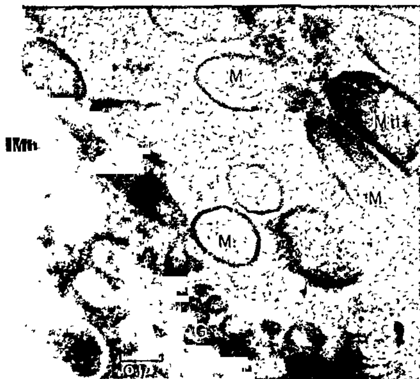


FIG 5 High magnification picture of albino melanin granules (M), note dense surface layer and faint strands of lamella material in their interior Mitochondria (Mit) and Golgi vesicles (G) Magnification × 106,667.

chondria are well-defined and distinct structures in these cells, and there is not the slightest evidence that melanin granules originate from them. Lerner *et al.* (1949), in the same tissue, found the bulk of the tyrosinase activity in the microsome fraction which would doubtless contain Golgi material but not intact mitochondria The concentration of mitochondria found around the formative zone of pigmented melanocytes, which is less conspicuous in albino melanocytes, suggests that mitochondria may nevertheless play some part in melanin formation.

In the melanocyte of the hair follicle, the Golgi material is the most



FIG 6 Low-power view of a melanocyte in European skin The field shows a melanocyte (M) with its long axis lying along the basement membrane (Bm) The cell contains filamentous mitochondria and a Golgi zone (G) in which early stages of melanin granules can be seen under high power Melanin granules in the melanocyte and Malpighian cells (top left) appear as dense ovoid structures Bundles of collagen (C) and a fibroblast (F) are seen in the dermis Magnification × 7200

probable source of the melanin granules. This is a series of parallel double membranes associated with vacuoles and lacking the adherent dense particles which characterize the endoplasmic reticulum (Dalton and Felix, 1957, Lacy and Challice, 1957). The cisternae of the Golgi material are often varicose, tending to separate into a series of vesicles or a reticulate sheet in three dimensions. Places may be found where vacuoles containing rudimentary melanin granules appear to be continuous with such a series of vacuoles. Frequently, the wall of a vacuole in which an immature granule lies is drawn out to a fine point as if it had been detached from adjacent Golgi material by constriction. Wissenfels (1956), however, studying cultured melanocytes from the silky fowl with phase contrast and electron microscopy, describes the melanin granules as arising in certain compact areas in the cell which he regards as distinct and separate from the Golgi apparatus

IV. The Granules of Red Hair

Under the light microscope the granules of strongly red hair are either very small or are not detectable. There are reasons for thinking that the pigment of red hair may be chemically different from melanin (Flesch and Rothman, 1945, Barnicot, 1956). Under the electron microscope the melanocytes in the follicles from subjects with strongly red hair contain ovoid granules 0.5 μ or less in diameter The structure of these is different from the granules in the melanocytes of other shades Sometimes they are hollow, with a layer of dense material forming an outer shell; more often they are composed of an aggregate of loosely packed dense subparticles. Granules of similar types can be detected in the keratinizing cortical cells and in fragments of mature cortical cells prepared by digestion of the hair in strong acid The presence of these granules, many of which would be barely detectable with the light microscope, does not exclude the possibility of additional diffuse pigment It is not known if granules of this kind are unique in red hair, or if similar types may also be found in blond non-red colors.

V. The Melanocytes of the Human Epidermis

Pease (1951) has published electron micrographs of human epidermal melanocytes, but his material affords little cytological detail Clear cells similar to those figured by Pease are occasionally found both in the basal layer of the epidermis, in contact with the epidermo-dermal junction, and at higher levels in the Malpighian layer The cell shown in Fig 6 has a tapered form with the long axis lying parallel with the basement membrane, the appearance of this cell follows the descriptions of melanocytes seen in detached sheets of epidermis

(Billingham, 1948; Billingham and Medawar, 1953). The cytoplasm of melanocytes lacks the bundles of keratinous fibrils found in the Malpighian cells. A differentiated area on one side of the nucleus consists of small vacuoles and occasional *double membranes, identifiable as the* Golgi material, mitochondria also occur in this area. A similar zone adjacent to the nucleus of another melanocyte is shown at high mag-

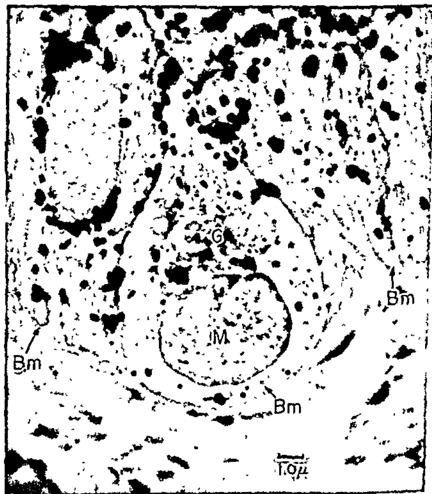


FIG 7 Low-power view of a melanocyte (M) lying on the basement membrane (Bm) of Negro skin The cytoplasm of the melanocyte contains no fibrillar material which distinguishes it from the epidermal cells on either side It contains a differentiated zone (G) on the side of the nucleus nearest the skin surface, this zone consists of Golgi material and mitochondria The cell is drawn out into processes pointing toward the Malpighian layer Magnification × 6750

nification in Fig. 7. Although dense, ovoid melanin granules are scattered throughout the cytoplasm, the zone in question also contains smaller dense bodies that resemble the formative stages of melanin granules described earlier in the melanocytes of hair follicles; this region, then, can be regarded as a zone of melanin formation. In other regions of the cell, fragments of endoplasmic reticulum are found. The melanin granules are about 0.3 μ or less in length and are thus smaller than those that occur in dark hair.

The melanocytes in the skin of Negroes are similar to those in the European, but the melanin granules that they form are more numerous and larger. In white or Negro skin the melanogenic zone of the melanocytes is readily identifiable, but it is smaller and less compact than that described in hair follicle melanocytes. This may indicate that the formation of melanin is less vigorous than in hair follicles. This is particularly evident in the melanocytes of Negroes, in which the concentration of melanin granules within the skin melanocytes is not as great as it is in those of the hair follicle. Melanin granules persist without apparent structural change in the stratum corneum In European white skin, the melanocytes have a restricted production of small but fully melanized granules, in the hair melanocytes of albinos numerous granules are formed, but they do not become melanized

The boundaries between adjacent epithelial cells are often difficult to distinguish, and do not show the conspicuous intercellular spaces containing obvious melanocyte dendrites. The periodic dense thickenings of the cell membrane of the epidermal cells along the junction with the dermis, as described by Selby (1955), are also present in the corresponding boundary of melanocytes, although they are less conspicuous.

Rounded cells with a nonfibrillar cytoplasm, mitochondria, fragments of Golgi material, and a few very small melanin granules are also found at higher levels in the Malpighian layer, and even in the stratum granulosum. The cells found in the stratum granulosum have only a finely granular cytoplasm and a much crenated nucleus. Such cells may be tentatively identified as the effete melanocytes, or Langerhans cells, described by Billingham and Medawar (1953)

VI Acknowledgments

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CHAPTER 13

The Nature of Hair Pigment[1]

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I. INTRODUCTION AND CLASSIFICATION OF HAIR AND FEATHER PIGMENTS

Although superficial examination of hair would indicate a wide range of color hues, microscopic examination has revealed only black, brown, and yellow pigmented granules.

[1] This investigation was supported in part by the Damon Runyon Cancer Fund, PHS Grant #CY-2408, and the Duke Laboratories, Stamford, Connecticut

Contributions by the geneticists on the role of various inherited traits as determinants of hair color have given the biochemist important leads. Differences in hair color are biochemical differences, and the genetic pattern of hair color indicates that brown and black pigment is under the same genetic control, whereas yellow (pheomelanin) is under a different genetic control. Thus two separate, but possibly interrelated, metabolic pathways of brown-black and yellow hair pigment are suggested.

Table I shows the most pertinent facts known at the present time regarding the classification of hair pigments The facts which are well

TABLE I
CLASSIFICATION OF HAIR AND FEATHER PIGMENTS

	Tyrosine-melanin[a] (Brown-black)	"Pheomelanin" (yellow-red)
Site of formation	Melanocyte	Melanocyte
Precursor	Tyrosine	*Tyrosine* *Tryptophan*
Enzyme	Tyrosinase	Tyrosinase *Enzymes involved in tryptophan metabolism*
Physical properties[b]	Brown or black granules Oval or round General absorption of ultraviolet	Aniline-yellow granules Round Exhibits fluorescence with 3660 A ultraviolet
Chemical properties	*Insoluble in dilute alkali* *Large polymer coupled with protein*	*Soluble in dilute alkali*

[a] Also known as "eumelanin"
[b] Partly from E S Russell (1946)

established are printed in regular type and the uncertain or hypothetical ones are printed in italics. The black-brown pigments are designated as "tyrosine-melanin" since they are formed in mammals by the enzymatic oxidation of tyrosine to melanin The yellow-red pigment, listed as "pheomelanin," has different solubility in alkali from the brown-black pigment Both tyrosine-melanin and pheomelanin are formed within the melanocyte and occur in the form of cytoplasmic granules Although tyrosine is generally accepted as the precursor of brown-black pigment (Table I), the origin of pheomelanin is unknown

A red pigment, first extracted from hair by Sorby (1878), was independently rediscovered, and named trichosiderin by Rothman and Flesch (1943). Trichosiderin appears to represent only a small portion

of pigments occurring with it in hair (Flesch and Rothman, 1945, Barnicot, 1956a, b). It has been extracted from the feathers of many races of fowl (Smyth *et al.*, 1951), but has not yet been extracted from the hairs of mammals other than man It has been suggested that trichosiderin is not a natural pigment but that it arises during acid hydrolysis of hair (Hanna, 1952).

In this discussion we will present evidence that tyrosinase is required for the formation of both tyrosine-melanin and pheomelanin. The precursor of pheomelanin could be tryptophan or a tryptophan metabolite, which is enzymatically oxidized to a yellow pigment only in the presence of intermediates of the tyrosine-melanin pathway.

II. The Inheritance of Hair Color

Color of hair in mammals is affected by a number of genes, of which twenty-four have been recorded in the mouse (Gruneberg, 1952), a number of them being pleiotropic, they may also affect other parts of the body in addition to affecting hair color. Color of hair depends on the chemical nature and on the quantity of pigment, and on the way in which it is distributed within the hair. Not much is known of the inheritance of hair color in man (Davenport and Davenport, 1909, Reed, 1952), although the genetics of hair color of laboratory rodents, domestic and farm animals are well understood. There appears to be a general uniformity in the complement of genes affecting color, one animal may have a gene which has no known counterpart in other animals, but on the whole there is a good deal of similarity in the complex of genes affecting hair color in different animals This account provides only an outline of the genetics of hair color to illustrate the points raised here. More detailed accounts of the genetics of mammals in general are found elsewhere (Castle, 1940, 1954, Wright, 1942, Russell and Russell, 1948, Gruneberg, 1952, Little, 1957).

In many mammals, but not man, hair is not uniformly colored, but it is barred with a terminal or subterminal band containing pheomelanin, and the base containing melanin Many rodents and lagomorphs have thus bicolored or "agouti" type of hair The genes at the agouti locus (*A,a* and several other allelomorphs) affect the hair in this respect, but usually a second pair of allelomorphs, the extension genes (*E,e*), must be considered in explaining the effects of the agouti genes

A guinea pig or rabbit with a dominant agouti gene, *A*, is able to produce a bicolored hair with pheomelanic tip and dark melanin base, if the dominant extension gene, *E*, is also present Animals whose genetic constitution contains *A*/—, *E*/— have agouti hair (Table II, 1), if the recessive extension genes, *e*/*e*, are present no dark melanin is

formed in the hair, and the hair is uniformly yellow (Table II, 2). With only recessive agouti genes, *a/a*, the hair is uniformly colored, being *melanic with E/—, and pheomelanic with e/e (Table II, 3 and 4).* Thus, with the dominant *A* gene, the tip of the hair is always pheomelanic while the base is pigmented with melanin or pheomelanin according to whether *E* or *e/e* is present. With the recessives *a/a*, hair is uniformly colored, and if *E/—* is present it is dark; if *e/e* is present it is yellow. The mouse has no *E* locus, and the presence of the A^y gene, top dominant in the agouti series, leads to the formation of uniformly pheomelanic hair (Table II, 5 to 7). It is of biochemical significance that a hair bulb can produce both melanin and pheomelanin pigment.

TABLE II

EFFECT OF AGOUTI AND EXTENSION GENES ON COLOR OF HAIR OF GUINEA PIGS (1-4) AND MICE (5-7)

Genotype	Color of tip	Color of base
1. *A/—, E/—*	Yellow	Dark
2 *A/—, e/e*	Yellow	Yellow
3 *a/a, E —*	Dark	Dark
4 *a/a, e/e*	Yellow	Yellow
5. A^y/—	Yellow	Yellow
6 A+/—	Yellow	Dark
7. *a/a*	Dark	Dark

If the genetic constitution of the animal leads to the formation of dark melanin in the hair, the shade of the pigment is influenced by genes at the black-brown locus. If *B/—* is present, the pigment will be blackish, if *b/b* is present the pigment is brown.

Hairs, whether colored by black or brown pigment or the yellowish pheomelanin, can show different intensity of color, and thus is influenced by a number of genes In albinos the dominant *C*, if present, potentiates intense pigmentation, at the other extreme, the recessives *c/c*, lead to whiteness of the hair, whatever is the nature of the pigment present or potentially present Several allelomorphs at the *C* locus are known from most animals, and these lead to intermediate intensities of pigmentation, but they do not reduce the intensity of melanin (if present) to the same extent that they reduce pheomelanin (if present) in animals differing only in this respect

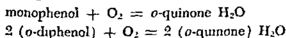
A number of other genes are known to affect the intensity of pigmentation, but only a few examples that have relevance in later discussions will be cited. In guinea pigs the genes, *P,p* (pink-eyed dilution) affect eye and hair color. animals with *P* have dark eyes and intense pigmentation, while those with *p/p* have pink eyes with melanin

(if present) diluted; pheomelanin is not diluted by p/p. A guinea pig with the dominant gene F and pheomelanic pigment is intensely colored, one with f/f has reduced pigment; melanin is unaffected Pigments may thus suffer dilution in connection with the presence of genes p/p, f/f, or those of the lower albino series, but the effect of the genes is different according to whether melanin or pheomelanin is present. These facts must be taken into account when the biochemistry of the process is considered.

Local failure of pigmentation is affected by the spotting genes; S,s, and s/s leads to formation of areas of white hair in the animal. A further gene at the extension gene locus, e^t (tortoise shell), leads to the production of localized areas of pheomelanic and melanic (or agouti) hair. Animals with e^t/e^t; s/s are tricolored, being white with patches of melanic and pheomelanic hair The presence of hairs of three colors in one animal has certain biochemical implications that will be discussed later.

III. Metabolic Pathway of Tyrosine-Melanin Formation

Melanins are among the most widely occurring pigments in nature and are responsible for the browning in plants, formation of the insect cuticle, and the tegumentary pigmentation of chordates. All melanins are formed by the action of a group of enzymes known as phenolases. The phenolases are copper-containing oxidases that catalyze the oxidation of mono- and *o*-dihydric phenols to *o*-quinones (Mason, 1956). The phenolases may, therefore, catalyze the incorporation of one atom of oxygen into a molecule of monophenol, or the oxidative dehydrogenation of diphenols to *o*-quinone.



$$\text{monophenol} + O_2 = o\text{-quinone } H_2O$$
$$2\ (o\text{-diphenol}) + O_2 = 2\ (o\text{-quinone})\ H_2O$$

The oxygen which is not incorporated into a monophenol is reduced directly to water There is no hydrogen peroxide formed.

The occurrence and the function of phenolase in many classes of organisms are summarized by Mason (1955) Mason (1956) suggests the use of the term "phenolase complex" in lieu of phenolase to emphasize the uncertainty of the nature of the active centers which catalyze the reactions in the above diagram In a panoramic survey of the phenolase complex, Mason (1955) cites its role in the formation of the intermediates in biosynthetic systems which produce the flower pigments and related flavonoids, the lacs and lacquers, the simple and polymeric tannins and their esters, the phenolic alkaloids, the quinones, tropolones and simple plant melanins, and the lignins The phenolase

complex catalyzes intermediate phases in cuticulation and in pigmentation of arthropods and other phyla In chordates, the phenolase complex catalyzes the oxidation of the phenol, tyrosine, to melanin which is localized in the skin, feathers, hair, scales, and eyes. "Each of the numerous heterotypic expressions of the phenolase complex is produced by a unique biochemical sequence which is characterized by (1) a phenolase specificity becoming narrower with rise in the phylogenetic scale, (2) a characteristic chemical position in a metabolic network, and (3) a specific physical localization within the cell and organ" (Mason, 1955)

The phenolase complex has reached the highest level of specificity in mammalian tissue where among the various phenols it catalyzes most readily the oxidation of tyrosine to melanin. Henceforth, the mammalian phenolase complex will be referred to as *tyrosinase*.

Knowledge of the biochemistry of mammalian tyrosinase has lagged behind studies of the other phenolase complexes present in plants and insects. Plant and insect phenolase complexes are available in large quantities, while mammalian tyrosinase is present in small amounts in tissues (skin and hair bulb) which are difficult to homogenize Most of the information of the biochemistry of mammalian tyrosinase has been obtained from studies of the enzyme present in the transplantable malignant melanoma of the mouse. Another difficulty in the study of mammalian tyrosinase is that the enzyme is attached to particles that are suspended in the cytoplasm of the melanocyte (Lerner *et al.*, 1949). It has not been possible until recently to prepare a soluble mammalian tyrosinase (Brown and Ward, 1957, Alben, 1957)

This brief introduction shows, in perspective, the place of mammalian tyrosinase in the spectra of phenolase complexes. The section that follows will present some of the facts known about mammalian tyrosinase, and the reaction sequence in which tyrosine is converted to melanin

A Outline of the Pathway of Tyrosine to Melanin

Melanin formation involves the conversion of a colorless, naturally occurring amino acid, tyrosine, to an insoluble brown polymer This process can be carried out in mammalian tissue only by the catalytic action of an enzyme The chemical reactions occurring in the transformation of tyrosine to melanin are summarized in Fig. 1 They were established by Raper (1928), who studied the aerobic oxidation of tyrosine and dihydroxyphenylalanine (dopa) in the presence of tyrosinase obtained from the meal worm, *Tenebrio molitor*. Mason (1948)

confirmed the findings of Raper studying tyrosinase from the malignant melanoma of the mouse.

Tyrosinase catalyzes tyrosine to dopa In the presence of tyrosinase, dopa is further oxidized to dopa quinone, which by an internal oxidation-reduction cyclizes to 5,6-dihydroxy-2,3-dihydroindole-2-carboxylic acid, this undergoes a nonenzymatic oxidation to the corresponding quinone, dopachrome (formerly incorrectly termed hallachrome). Dopa-

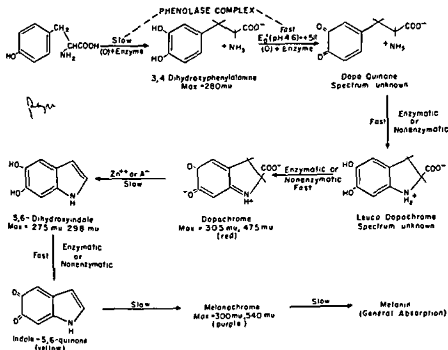


FIG 1 Enzymatic oxidation of tyrosine to melanin.

chrome, also by internal oxidation-reduction, loses carbon dioxide, forming 5,6-dihydroxy-indole, the immediate precursor of melanin The polymerization of the monomer, 5,6-dihydroxyindole to a large polymer (melanin) occurs first, by oxidation to indole-5,6-quinone and subsequently polymerization through the 3-, 4-, 7-, and occasionally 2-, positions to give a polymer depicted in Fig 2 (Mason, 1955). The melanin polymer is attached to protein through its quinone linkages. It is not clear whether the quinone of the melanin polymer is attached to the amino or sulfhydryl groups of protein Melanin in mammalian tissue is always attached to protein

It is likely that the scheme of Raper (1928) operates in the human

melanocyte, although all of the intermediates have not been isolated from the system during enzymatic catalysis by tyrosinase. The chromogen in human melanuria, however, is a mixture of conjugated derivatives of 5,6-dihydroxyindole (Dalgliesh, 1955).

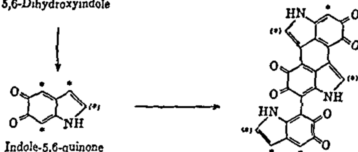


FIG 2 Suggested polymerization in melanin formation Asterisks indicate sites of coupling of monomers (After Dalgliesh, 1955)

B. *Nature and Mechanism of Action of Mammalian Tyrosinase*

Although an enzyme hypothesis was postulated over 60 years ago for the formation of mammalian melanin, it was not until 1942 that Hogeboom and Adams demonstrated with modern biochemical techniques the presence of tyrosinase in crude preparations of Harding-Passey mouse melanoma These authors reported a partial separation of monophenolase (enzymatic action in the conversion of tyrosine to dopa) and diphenolase (*enzymatic action in the oxidation of dopa to* dopa quinone) activities With the demonstration of tyrosinase and "dopa oxidase" these authors revived the long-standing controversy of the *one* (tyrosinase) *or two* enzyme (tyrosinase and a separate enzyme, dopa oxidase) hypotheses of melanin formation. The solution to this enigma now appears at hand Lerner *et al* (1949) were unable to show a separation of tyrosinase from dopa oxidase activities Using ammonium sulfate precipitation, fractional ethanol precipitation and differential centrifugation, they noted during the fractionation procedures a progressive increase in the induction or lag period in the oxidation of tyrosine. The dopa oxidase activity of Hogeboom and Adams (1942) *was tyrosinase activity with a prolonged induction period* and no apparent action on the oxidation of tyrosine In the presence of catalytic amounts of dopa, the induction period was shortened linearly according to the logarithm of added dopa concentration Thus, Lerner *et al.* (1949) confirmed in mammalian tissue the already well-established phenomenon of the phenolase complex in plants A synthesis of opinion

and a new one-enzyme hypothesis of the phenolase (tyrosinase) and diphenolase (dopa oxidase) activities has recently been summarized by Mason (1956). Both catalytic activities (phenolase and diphenolase) have been shown to occur together and are associated with the same electrophoretic and ultracentrifugal components. In any given preparation of the phenolase complex, both phenolase and diphenolase are inhibited to the same degree by metal-binding agents or competitive inhibitors Phenolase and diphenolase activity are proportional to the content of copper, and enzymatic activity is lost when the copper is removed. It has not been possible to remove diphenolase activity from phenolase activity in any given preparation. The oxygen tracer studies reported by Mason *et al.* (1955) have shed new light on the mechanism of action of tyrosinase and some clear ideas have been formulated on

ACTIVATION: Protein — Cu^{++}_2 + Dopa ⇌ Protein — Cu^{+}_2 + Dopa Quinone + H_2O

O_2 COMPLEX FORMATION: Protein — Cu^{+}_2 + O_2 = Protein — Cu^{+}_2 — O_2

MONOPHENOL OXIDATION. Protein — Cu_2 — O_2 + Tyrosine = Protein — Cu^{+}_2 + Dopa Quinone + H_2O

FIG 3 Mechanism of action of tyrosinase (After Mason, 1956)

the manner in which a single enzyme is able to catalyze the first two reactions in the scheme of Raper (1928) (conversion of tyrosine to dopa and dopa quinone) In the presence of dopa, two cupric atoms in tyrosinase are reduced to cuprous atoms. In the absence of dopa or other reducing agents, hydroxylation by tyrosinase does not occur. Thus the initial step in the action of tyrosinase is a reduction of the enzyme from the cupric to the cuprous state Dopa, in addition to acting as substrate, can act as an activator for reduction of the cupric to the cuprous state of tyrosinase (Fig 3) *In vivo*, tyrosinase may exist as a reduced enyzme active toward both tyrosine and dopa, or, because of a high prevailing oxidation potential, may exist as an oxidized form in which tyrosinase is inert but activable toward tyrosine and readily active toward dopa.

Alben (1957), using the tyrosinase present on melanin granules from the Harding-Passey mouse melanoma, found a single enzyme which may catalyze the oxidation of either tyrosine or dopa Mammalian tyrosinase is relatively specific for the L-configuration, in the oxidation of dopa and in the activation by dopa of tyrosine oxidation

Factors which change the rate of enzymatic dopa oxidation alter the rate of activation (induction period) and the maximum rate of oxygen consumption in the presence of L-tyrosine. The rate of activation appears to be related to the rate at which dopa may be enzymatically oxidized. Thus, the induction period in the oxidation of tyrosine by tyrosinase is prolonged as the concentration of the enzyme is decreased. It will be shown later that this inverse relationship of the induction period and the enzyme concentration may offer an explanation of the histochemical studies which show a difference of tyrosinase and dopa oxidase activity in the melanocytes of the hair bulb and epidermis.

C. Localization of Tyrosinase in the Melanocyte

The metabolic unit of melanin formation in the melanocyte is the melanin granule. These protein granules contain, in addition to tyrosinase, at least two other enzymes, cytochrome oxidase and succinic dehydrogenase (Herrman and Boss, 1945). The granules appear to develop in specific pigment formation centers from cytoplasmic vesicles by the deposition of concentric layers of melanin (Birbeck *et al*, 1956) Structurally, melanin granules are rounded and regular in shape, about 0 15 to 0 4 μ in human skin (Barnicot *et al.*, 1955). Melanin granules *present in the retinal pigment epithelium of the chick are much larger* and vary from 1 0 to 3.0 μ in diameter (Miyamoto and Fitzpatrick, 1957b) Melanin granules isolated from brown or black human hair vary considerably in size and shape (Barnicot *et al*, 1955), they exist as slender rods and in some instances appear circular. Under the electron microscope, areas of lower density near the center of the granule give them a "ring" shape. *In electron micrographs a colorless "pre-*melanin" core can be seen, upon which the polymerized derivative of oxidized polyphenols is deposited as a sheath or envelope (Laxer *et al*, 1954) During melanization successive layers of melano-protein appear to be deposited on the surface of the granule In the formation of melanin "cuticle," tyrosinase activity may become successively less detectable because the enzyme is inactivated by a process similar to "tanning."

The relationship of the melanin granule to mitochondria is not clear at this time Although melanin granules can be stained with Janus green B, they are structurally dissimilar to mitochondria The ergastoplasm and mitochondria of the melanocyte appear to be similar in structure to those in other cells (Barnicot *et al*, 1955, Birbeck *et al*, 1956)

The pigment-forming ability is possibly spread by the transfer of "some cellular ingredient" from the cytoplasm of a pigmented melanocyte to the cytoplasm of a nonpigmented one, endowing the latter with

the capacity to form melanin (Billingham and Medawar, 1948). This "cellular ingredient" may be cytoplasmic, metabolically active melanin granules. Since the melanin granules of the hair are contained within the compact, isolated unit of the hair follicle there is no possibility of "pigment spread" from one hair bulb to another. The transfer of melanin granules from cytoplasmic processes of hair melanocytes to the cortical cells has been observed to occur in the form of rounded bundles closely packed with granules which disperse as the cortical cells elongate and keratin fibrils form (Birbeck *et al.*, 1956).

D. *Factors Regulating Melanin Formation (in Vitro)*

The stimulus for the rather extensive investigation of inhibitors of melanin formation is largely the result of attempts to provide a chemo-

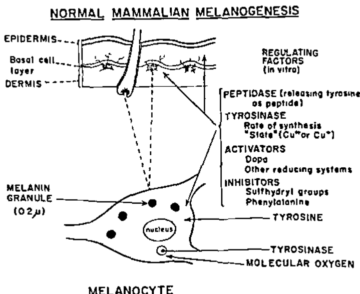


FIG. 4. Factors controlling melanin formation.

therapeutic approach to the treatment of the malignant melanoma. Although melanin formation may not be related to the malignant proliferation of melanocytes, the limitation of the tyrosine-melanin pathway to the melanocyte may serve as a means of localizing mitotic inhibitors or destructive radioactive elements. The regulation of melanin formation is dependent on at least four factors (Fig. 4).

1. The Availability of the Melanin Precursor, Tyrosine

Mammalian tyrosinase cannot catalyze the oxidation of tyrosine bound in a peptide link through its amino group (Lerner *et al.*, 1951). In order for melanin formation to occur, free tyrosine must be made available. Miyamoto (1957) has examined C-terminal peptides of L-tyrosine (L-leucyl, D-leucyl and glycyl) as substrates of homogenates of embryonic chick retinal pigment epithelium. Only L-leucyl-L-tyrosine and glycyl-tyrosine were oxidized in the presence of the enzyme. Paper chromatographic analysis of the reaction mixtures revealed that these dipeptides split into amino acids, and that the amino acids, except for L-tyrosine, remained unchanged. The presence of these peptidases in association with tyrosinase is suggestive of their role in melanin formation, since the melanocyte probably contains tyrosine bound by a peptide linkage rather than as free tyrosine.

2 The Rate of Tyrosinase Synthesis

When concentrations of tyrosinase are low, there is a prolonged induction period in the conversion of tyrosine to dopa. In human skin, tyrosinase is present in low concentrations and histochemical studies have shown that although the conversion of dopa to melanin occurs (Bloch's dopa reaction), tyrosine is not converted to melanin during the period of incubation (Fitzpatrick *et al.*, 1950, Fitzpatrick, 1952) In the malignant melanoma (Fitzpatrick and Kukita, 1956) and in the melanocytes of the brown or black human hair bulb (Kukita and Fitzpatrick, 1955), tyrosinase concentration is apparently high since there is a rapid conversion of both tyrosine and dopa to melanin It is not clear, however, whether the relatively rapid conversion of the tyrosine to melanin by tyrosinase present in the brown or black hair bulb melanocytes is related to an increased rate of melanocyte proliferation, and *pari passu* increased concentrations of tyrosinase, or is related to the activation of an inhibited tyrosinase during the hair growth cycle

3 Presence of Factors Which Activate Tyrosinase

The presence of dopa or other reducing agents provides the initial "priming" step in the hydroxylation of tyrosine and thus the initiation of the tyrosine to melanin metabolic pathway. It is unlikely that dopa, which is very unstable, can exist *in vivo* in the absence of other reducing agents such as ascorbic acid *In vitro*, ascorbic acid can function in a dual manner since it increases the rate of conversion of tyrosine to dopa, and it allows the accumulation of dopa by reducing dopa quinone to dopa (Lerner and Fitzpatrick, 1950). Since L-dopa is relatively specific

in its action as an activator of tyrosinase (Alben, 1957), it is perhaps the most important metabolite in the initiation of the tyrosine to melanin reaction. However, dopa can only be formed from the hydroxylation of tyrosine by tyrosinase, some other chemical reaction must provide reducing agents that accomplish the reduction of cupric-tyrosinase to cuprous-tyrosinase in order for dopa to be formed initially. Once dopa has been formed, it can act to reduce cupric-tyrosinase to cuprous-tyrosinase

4. Presence of Inhibitors of the Tyrosine-Melanin Pathway

A large number of chemicals inhibit melanin formation *in vitro* (Lerner and Fitzpatrick, 1950, 1953). These include substances that inhibit competitively (3-fluorotyrosine, *N*-acetyltyrosine, *N*-formyltyrosine, and phenylalanine and its degradation products) and a fairly large group of chemical agents that combine with copper prosthetic group of tyrosinase phenylthiourea, diethyldithiocarbamate, 2,3-dithiopropanol, cysteine, glutathione, etc.). Chemicals that alter the redox potential hydroquinone, phenol indophenol) may also inhibit melanin formation. Finally, aminophenyl compounds such as aniline, 3-aminotyrosine, and *p*-phenylene diamine can inhibit melanin formation by combining with one of the intermediates such as dopaquinone.

Despite a considerable knowledge of *in vitro* inhibitors of the tyrosine-tyrosinase reaction, there is very little knowledge of the factors which regulate melanin formation *in vivo* by inhibiting the tyrosine-melanin pathway

The role of sulfhydryl groups in the regulation of melanin formation has been studied by Rothman *et al.* (1946). His hypothesis, based on *in vitro* studies, states that tyrosine and an active functioning tyrosinase in melanocytes are unable to react because of the presence of inhibitory sulfhydryl groups Since Rothman used epidermal extracts as a source of sulfhydryl groups, it is not clear whether the inhibition of the tyrosine-tyrosinase reaction was related to the sulfhydryl groups contained within melanocytes, or in the epidermal cells which are rich in sulfhydryl groups. Using histochemical techniques, it has not been possible to "activate" the normally inhibited tyrosinase of human skin with sulfhydryl poisons (iodoacetamide, *p*-chloromercuribenzoate, etc.) (Fitzpatrick, 1952) Some recent studies to be discussed below on the changing concentrations of sulfhydryl groups and tyrosinase during the hair growth cycle, however, provide at least circumstantial evidence that sulfhydryl groups may be involved in the regulation of tyrosinase activity in the hair bulb

The possible role of phenylalanine and its degradation products in

the regulation of *in vivo* melanin formation will be discussed below in the section devoted to biochemical factors that regulate melanin formation in hair.

The possible interplay of the four regulatory factors described above, i.e the availability of tyrosine, the rate of tyrosinase synthesis, the presence of activators or inhibitors of tyrosinase is illustrated in a study of melanin formation in the developing retinal pigment epithelium of the embryonic chick (Black Australorp and Rhode Island Red) (Miyamoto and Fitzpatrick, 1957b). In this system there appears to be a sensitive regulatory mechanism of tyrosinase activity during various stages of development During the growth phase of the retinal pigment epithelium, there is a gradually increasing tyrosinase activity that ultimately achieves a QO_2 with tyrosine equal to that of the malignant melanoma On the twelfth day of development, when one would have expected a more gradual fall in activity, there is a curious sudden cessation of tyrosinase activity. This sudden change in tyrosinase activity can best be explained by the appearance of an inhibitory factor that blocks tyrosinase activity. A similar cyclic change in tyrosinase, with sudden cessation of activity, is present in the hair growth cycle of the mouse

IV CYTOCHEMICAL STUDIES OF TYROSINE-MELANIN FORMATION IN HAIR

One of the goals of modern biochemical research is to relate biochemistry to morphology, to unravel the intricacies of the intermediary metabolism, and to locate the specific organ, cell, or cell fraction in which they occur. The problem of melanin formation is both biochemical and morphologic, for although the biochemical conversion of tyrosine to melanin in an extract or homogenate has been fully worked out, an understanding of the process of melanin formation as it occurs in the melanocyte requires cytochemical studies.

Tyrosinase present in extracts of human malignant melanomas is identical to the tyrosinase present in the mouse malignant melanoma (Kertesz, 1954). However, progress in elucidating the mechanism of melanin formation in *normal* melanocytes has been hampered by technical problems. The tyrosine-tyrosinase reaction is ideally suited for a cytochemical technique Tyrosine, a water-soluble, stable amino acid, attaches itself to tyrosinase, and the oxidation and polymerization to insoluble melanin occurs on the surface of the melanin granule. Diffusion of substrate is not possible since tyrosinase is bound to the cytoplasmic granule of the melanocyte, and a second step is not required to visualize the end product Furthermore, the conversion of water-soluble tyrosine to insoluble melanin presents a unique opportunity for

utilization of an *in vitro* radioautographic technique. The unreacted, water-soluble labeled tyrosine is washed out of the tissue, and it is then possible to record on the photographic emulsion only the newly formed radioactive melanin.

The use of Bloch's histochemical "dopa-oxidase" reaction (1921) in the study of melanin formation has serious limitations. Dopa is an unstable amino acid and is nonspecifically oxidized to melanin by a variety of enzymes and oxidizing systems other than tyrosinase For example, dopa is converted to melanin by the oxidase in leucocyte granules. Tyrosine, on the other hand, is oxidized to melanin only by tyrosinase contained in the cytoplasm of melanocytes. The use of dopa as a substrate for tyrosinase in melanocytes is valuable when low concentrations of the enzyme are present, as in normal human epidermal melanocytes As mentioned previously, when tyrosinase is present in low concentrations, the induction period in the oxidation of tyrosine, but not dopa, to melanin is markedly prolonged.

A. *The Melanocyte System*

The only site of melanin formation in the mammal is the pigment cell or *melanocyte* (Billingham, 1949, Becker *et al*, 1952) This is a specialized, distinctive cell characterized by two or more dendritic processes and containing in its pigmented form myriads of brown cytoplasmic granules. In man, melanocytes are present in relatively few sites: at the epidermal-dermal junction, rarely in the dermal connective tissue, in the upper part of the hair bulb, in the leptomeninges, the uveal tract, and in the retinal pigment epithelium. In other mammals, birds and frogs, melanocytes may occur in many sites. In the house mouse (*Mus musculus*) melanocytes may occur in the nictitans, harderian gland, parathyroid, and thymus, in addition to melanocytes in locations similar to man (Markert and Silvers, 1956). Two distinct types of melanocytes (Markert and Silvers, 1956) are present in the mouse: (1) *Nucleopetal*, containing long, relatively thick, cytoplasmic processes with melanin granules more or less concentrated in the perikaryon In man, the nucleopetal type of melanocyte occurs in the dermis, uveal tract, and leptomeninges, (2) *Nucleofugal*, with more numerous long dendritic processes, and melanin granules more evenly distributed throughout the perikaryon and dendritic processes In man, nucleofugal types of melanocytes occur in the bulb of black, brown, and red hair and the epidermal-dermal junction

Because of the similar cytologic and cytochemical properties of melanocytes in the various sites mentioned above, it appears reasonable to group all the melanocytes under the designation, *melanocyte system*

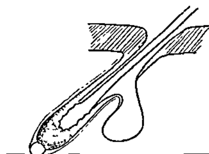
bers of melanocytes are found in the hair bulbs, and both tyrosinase and *dopa oxidase activity are high:* *Twenty-four days after plucking (telogen)* tyrosinase and dopa oxidase activity are not detectable The results are shown in Figs 5 to 10 and Table IV.

TABLE IV
CHANGES IN TYROSINASE AND DOPA OXIDASE ACTIVITY IN HAIR GROWTH CYCLE OF C57 BLACK MOUSE

Stages of hair cycle	Tyrosinase *activity*	Dopa oxidase *activity*	
Anagen I (proliferative phase)	Negative	Negative	Melanocytes not identified
Anagen II	Negative	Negative	Melanocytes not identified
Anagen III	Weakly positive	Positive	Melanocytes present in hair bulb
Anagen IV	Positive	Positive	Melanocytes present in hair bulb
Anagen V	Strongly positive	Strongly positive	Melanocytes present in hair bulb
Anagen VI	Strongly positive	Strongly positive	Melanocytes present in hair bulb
Telogen (resting phase)	Negative	Negative	Melanocytes not identified

The melanocytes in the hair bulb seem to be self-perpetuating and to undergo mitosis like other cells in the bulb (Montagna, 1956) The appearance of tyrosinase activity during anagen III to VI could reflect either a proliferation of melanocytes, with increased concentrations of active tyrosinase, or the activation of tyrosinase in existing melanocytes of the hair bulb. The latter explanation presupposes the establishment of an optimal biochemical milieu for tyrosinase activity in the developing hair bulb. Since the presence of tyrosinase activity coincides with the appearance of melanocytes in anagen III (Fig 3) and the enzymatic activity becomes more strongly positive with the increasing melanocyte population during anagen III to anagen VI, it is more likely that the increase in tyrosinase activity is associated with the proliferation of melanocytes

A number of enzymes have been shown to vary with the various *phases of the hair cycle (Montagna, 1956), but their relationship to* melanin formation is not clear The level of tyrosinase activity parallels that of succinic dehydrogenase activity during the hair growth cycle



A

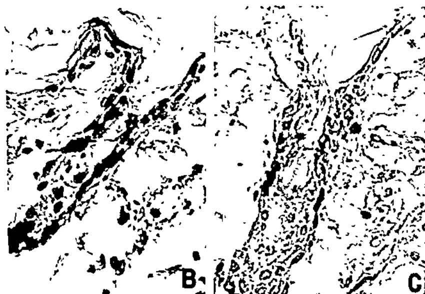
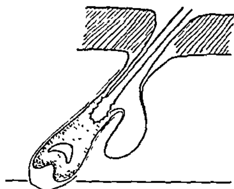


Fig. 5. Anagen I. At this stage mitotic activity begins. (A) diagram of phase. (B) Hematoxylin and eosin. Magnification × 400. (C) Radioautograph. No tyrosinase activity is present. Lithium carmine. Magnification × 400



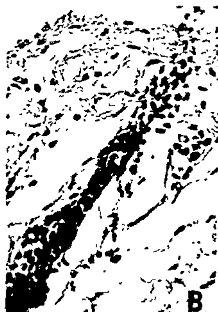
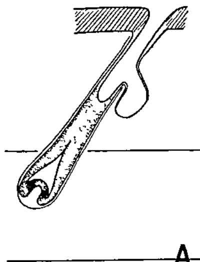




FIG 6 Anagen II (A) The "germ" grows down around the papilla and keratinization in the internal sheath occurs (B) Hematoxylin and eosin Magnification. × 400 (C) Radioautograph No tyrosinase activity has yet appeared Lithium carmine Magnification × 400



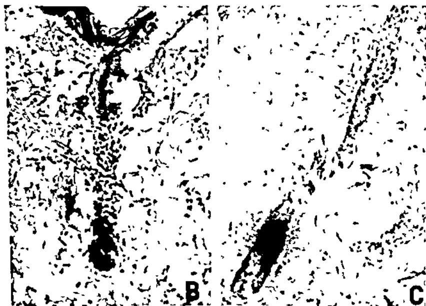


Fig 7 Anagen III As the result of mitotic growth in the external sheath and the "germ," the follicle reaches its maximum length and the bulb is fully formed (A) Melanocytes appear, (B) and reside along the papilla cavity and melanin formation begins (Hematoxylin and eosin Magnification, × 400), (C) as evidenced by weak tyrosinase activity Radioautograph Lithium carmine. Magnification × 400

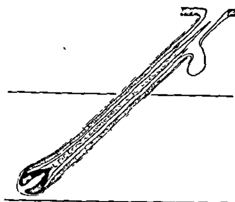






FIG 8 Anagen V The tip of the hair has grown to the level of the epidermis, the bulb attains its typical shape (A) and both the upper portion of the bulb and the hair shaft are pigmented, (B) Hematoxylin and eosin Magnification × 400 Marked tyrosinase activity is present in the region of the bulb (C) Radioautograph Lithium carmine Magnification × 400

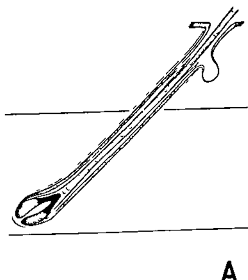




FIG 9 Anagen VI The hair has emerged beyond the surface on the skin (A) and (B) Hematoxylin and eosin Magnification × 400 There is a marked tyrosinase activity, (C) Radioautograph Lithium carmine Magnification × 400

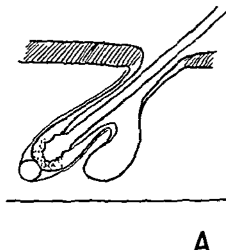






FIG 10 Telogen The matrix-cell proliferation has ceased, and a hair has formed The "germ" or cluster of cells is located just beneath the club Melanin granules can be seen in "germ" region (A) and (B) Hematoxylin and eosin. Magnification × 400 Melanin formation actually ceases abruptly at the catagen stage Although nonpigmented melanocytes are present in the germ, and presumably contain tyrosinase, there is no evidence of tyrosinase activity (C) Radioautograph Lithium carmine Magnification × 400 The biochemical factors responsible for the "block" of tyrosinase activity during telogen and anagen I and II are not known It is possible that tyrosinase is present in such low concentrations that it cannot be detected, or the enzymatic activity may be masked by the presence of an inhibitor which is present during early anagen, catagen, and telogen

Increased succinic dehydrogenase activity is found only during the proliferative (anagen) phase of the hair cycle (Argyris, 1956).

Changes in the sulfhydryl groups during the hair growth cycle are possibly also involved in melanin formation, and Rothman (1954) has postulated their role in the control of tyrosinase activity. During early anagen, when tyrosinase activity is negative, the hair bulb is rich in sulfhydryl groups In later anagen, when tyrosinase activity becomes strongly positive, the concentration of sulfhydryl groups is localized predominantly to the keratogenous zone which is far above the locale of the melanocytes. Montagna (1956) has emphasized, however, that free sulfhydryl groups, not demonstrated by histochemical techniques, may also be present in the growing hair bulb and glutathione (a tyrosinase inhibitor) may be abundant in the matrix cells since it is important in cell division.

Which of the four factors regulating melanin formation mentioned previously (availability of tyrosine, rate of tyrosine synthesis, presence of activators, and presence of inhibitors) are operating in the changes of tyrosinase activity during the hair growth cycle is not known at present. The rate of tyrosinase synthesis may parallel the rate of hair bulb melanocyte proliferation. Activation of tyrosinase by reducing systems formed during the proliferative phases of the hair growth cycle may provide the initial "priming" step in the hydroxylation of tyrosine by reducing cupric-tyrosinase to cuprous-tyrosinase The most interesting facet of this problem of hair bulb tyrosinase regulation is the possibility of an inhibiting substance which changes in concentration during the hair growth cycle Montagna (1956) has noted that melanin formation is inhibited shortly before hairs cease to grow, "as if the increasing titer of this substance were first detected by sensitive pigment cells." Many important leads to factors regulating melanogenesis may be found in a study of the various chemical changes occurring in the hair bulb during the growth cycle A biochemical approach to this problem is probably not feasible because of the difficulty of obtaining large quantities of hair bulbs The application of the Cartesian diver to the measurement of enzymatic changes in the hair bulb during the hair growth cycle may provide quantitative data.

The melanocytes of the hair bulb are the most rapidly proliferating pigment cells in the mammal, yet, their division must be rigidly controlled since malignant proliferation of hair bulb melanocytes has not been found Thus, the elucidation of the biochemical factors controlling melanocyte proliferation in hair bulbs may ultimately lead to the development of chemical agents which inhibit the growth of melanoma cells.

D. The Effect of Genetic Factors on Tyrosinase Activity

The association of oxidative enzymes with melanic pigmentation was first demonstrated by Bertrand (1896). Many investigators have since then studied the relative ability of skin and hair follicles of different colors to oxidize tyrosine and dopa. Durham (1904) showed that pigment was formed when extracts of skin of black rabbits, guinea pigs, and rats, and of yellow guinea pigs were incubated with tyrosine. Onslow (1915) found that this occurred with extracts of skin of black and brown, but not yellow, rabbits, extracts from recessive white rabbits failed to form pigment, and extracts from dominant whites inhibited pigment formation if added to black skin extract and tyrosine. Onslow included ferrous sulfate, and Durham included hydrogen peroxide in the incubation media, so that the meaning of their results is unclear (Pugh, 1933, Charles, 1938)

Frequently negative results have been obtained with tyrosine as substrate (Russell, 1939, Ginsburg, 1944), and dopa has been customarily used as substrate for the oxidative enzyme system The use of tyrosine was reintroduced by Fitzpatrick *et al* (1950) using human skin and by Foster (1951) using the skin of mice The dopa reaction has been carried out with the tissues of rabbits, guinea pigs, and mice and the oxidative activity was measured by the amount of pigment formed by extracts of skin (Ginsburg, 1944), by the amount of pigment deposited in hair follicles in histological sections (Russell, 1939, Foster, 1952a, Foster and Cook, 1954), and by manometric methods (Foster, 1951, 1952a, b, 1956a, b) In spite of some differences in details these results show a good measure of agreement. Follicles of white-haired guinea pigs, in both c^a/c^a albinos, and in the white-spotted areas of s/s animals, give a negative dopa reaction (Schultz, 1925, Kroning, 1930, Russell, 1939, Ginsburg, 1944) Melanics, $E/—$, and yellows, e/e, give the dopa reaction, usually the reaction has been found to be weaker in yellows In brown, b/b, melanics the dopa oxidase activity is not very different from blacks, $B/—$ Intensely pigmented animals, $C/—$, show a stronger dopa reaction than animals having other genes of the albino series present The effect that the presence of lower members of the albino series has upon oxidase activity differs according to whether genes $E/—$, for melanism, or e/e, for yellow hair, are present (Russell, 1939, Ginsburg, 1944). Of the other genes that dilute pigmentation, f/f gives a weaker dopa reaction than $F/—$; the reaction with $P/—$ or p/p is similar.

The amount of pigment naturally present in the hair of animals of different genetic constitutions has been studied by Russell (1938),



FIGS 11–16 Radioautographic tyrosinase reaction in hair bulbs of different genotypes in anagen VI

FIG 11 Intense brown (*a/a, b/b, C/C, D/D, P/P*). (A) Unstained Magnification × 700 (B) Radioautograph Note complete obliteration of hair bulb by silver grains This genotype had the most marked tyrosinase activity in this series. Lithium carmine Magnification × 700.



FIG 12 Brown with Maltese dilution (*a a, b/b, C/C, d d, P P*) In this genotype the number of melanin granules is the same as in the brown but the granules are clumped (A) Note clumping of melanin granules Unstained Magnification × 700 (B) Radioautograph with almost equal intensity of the tyrosinase reaction as the intense brown Lithium carmine Magnification × 700

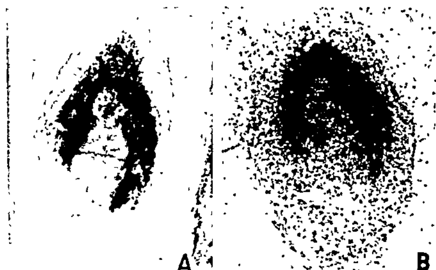


FIG 13 Yellow (A^y/a, B/B, C/C; D/D, P/P) (A) Unstained Magnification × 700 (B) Radioautograph Note that the intensity of the reaction is much less than in the brown and brown with Maltese dilution genotypes Lithium carmine. Magnification. × 700



FIG 14 Black (a/a, B/B, C/C, D/D). (A) Unstained Magnification × 700 (B) Radioautograph The tyrosinase activity is marked but *not* as intense a reaction as in the brown genotype Lithium carmine Magnification × 700

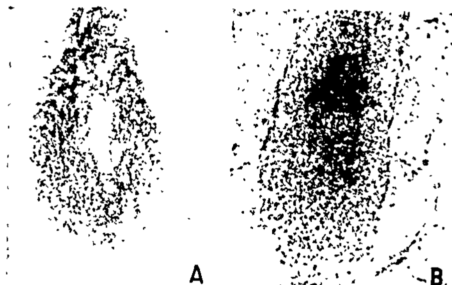


FIG 15 Black with pink-eyed dilution (*a/a, B/B, C/C, D/D, p p*). (A) Unstained Magnification × 700. (B) Radioautograph. There is less intense tyrosinase activity than in any one of the other genotypes including the yellow. Lithium carmine *Magnification* × *700*

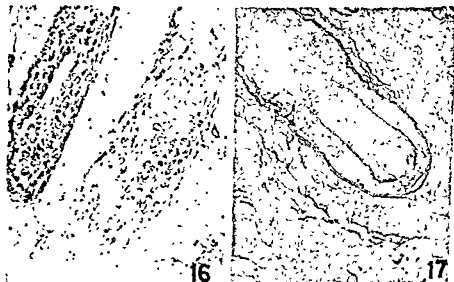


FIG 16 Albino (*a a, B/B, c/c, D/D, P P*) Radioautograph There is no tyrosinase activity *The few scattered silver grains are artifacts Lithium carmine* Magnification × 700

FIG. 17 Human albino scalp hair Radioautographic tyrosinase method No deposition of silver grains is found in the region of the upper matrix indicating an absence of tyrosinase activity Lithium carmine Magnification × 400

than pheomelanic ones. The activity is lessened by the presence of the lower membranes of the albino series, and by the recessive genes, *f/f*. Genes at loci *C* and *F* appear to control directly the activity of tyrosinase. It is neither understood why the activity is greater in melanic than in pheomelanic follicles, nor why the dilution genes have an effect on tyrosinase activity, which does not correspond with the amount of naturally occurring melanin found in the hair of animals with these genes

V. Biochemical Basis of Changes in Hair Color

With the present knowledge of melanogenesis it is possible to explain only a few of the numerous examples of the decrease or absence of melanin in hair (achromotrichia). Dietary deficiency of certain vitamins produce achromotrichia (Frost, 1948). However, none of these have any known effect on the tyrosine-tyrosinase reaction. In the present discussion only examples of achromotrichia in which the biochemical lesion is known will be reviewed

A. *Enzyme (Tyrosinase) Deficiency*

1. *Genetic*

The production of enzymes is controlled by genes, and some biochemical geneticists believe that a single gene controls the synthesis of only one enzyme. Total albinism, an inherited character in many animals, is found where only the lowest member of the albino series of genes is present In total albinos, no tyrosinase activity has been detected in hair follicles (Fig 17) Usually there is a number of allelomorphs at the C-locus, each one being correlated with a degree of tyrosinase activity, *C/*— facilitating intense pigmentation, other members facilitating dilute pigmentation, and *c/c* which is associated with albinism.

Localized pigmentary deficiencies may also occur ranging from large patches of white on the body to the white forelock often found in man. A number of genes, both dominant and recessive, control these effects. Some, such as spotting in guinea pigs, are due to local absence of tyrosinase, other cases, such as in the "English" strain of rabbits are said to be partially white as a result of local inhibition of melanogenesis

The inability to form melanin in the skin and hair bulb of the albino is caused by the absence of the enzyme, tyrosinase, and not by the absence of melanocytes (Lerner and Fitzpatrick, 1950). Barnicot *et al* (1955) have identified melanocytes in the human albino hair bulb with the aid of electron microscopy.

2 *Nutritional Deficiency (Copper)* •

Since copper is the prosthetic group of tyrosinase, absence of this trace element in the diet results in a loss of enzyme action as the apoenzyme cannot catalyze the oxidation of tyrosine to melanin Copper-deficient diets invariably result in achromotrichia in rats (Keil and Nelson, 1931, Free, 1940), cats (Gorter, 1935), rabbits (Gorter, 1935, Smith and Ellis, 1947), and cattle (Sjollema, 1937). Further evidence that copper is essential in melanogenesis is provided by the development of achromotrichia in chronic molybdenum (Muir, 1941). Cattle grazing on pastures containing excessive amounts of molybdenum develop achromotrichia, this is overcome by the adminstration of copper sulfate.

3 *Ageing*

Bloch (1921), who studied the dopa oxidase reaction in human hair bulbs, noted that in ageing subjects with gray hair there was a markedly diminished melanin formation in the hair bulbs after incubation in dopa. In elderly subjects with white hair he found no melanin formation after *incubating the hair bulbs in dopa. Bloch concluded that the intensity* of the histochemical dopa reaction could be correlated with the varying amounts of melanin pigment present in the hair bulb. This conclusion has essentially been confirmed in a study of tyrosinase activity in human hair bulbs, using the histochemical radioautographic tyrosinase method. Tyrosinase activity of melanocytes present in the hair bulb of black human hair is illustrated in Fig 18 The radioautograph of a hair bulb from a gray-haired subject is illustrated in Fig. 19. The absence of silver grains in the hair bulb indicates a deficiency of tyrosinase No tyrosinase activity was detectable by the radioautographic tyrosinase method in hair bulbs from seven additional gray-haired subjects. Dopa is oxidized to melanin even in the presence of low levels of tyrosinase, a positive dopa reaction in the ageing *gray* hair bulb and a negative dopa reaction in the *white* hair bulb suggests a gradual loss of enzymatic activity.

In summary, the graying of hair associated with senescence appears to be the result of a gradual loss of tyrosinase activity of hair bulb melanocytes It is possible that graying may develop as a result of tyrosinase inhibition by metabolites that accumulate in the hair bulb during senescence.

Graying of hair never precedes, but always follows melanin pigmentation and thus must be considered a manifestation of melanocyte ageing. The loss of tyrosinase activity in the gray hair bulb, whether caused by failure of enzyme synthesis or inhibition of enzymatic activ-

ity, is perhaps the most constant biochemical lesion yet observed in senescence.

B. *Enzyme (Tyrosinase) Inhibition*

1 *Competitive*

Since enzymes are only relatively specific, they can combine reversibly with substrates which do not undergo a reaction and thus act as competitive inhibitors by blocking the active site of the enzyme. An example of competitive inhibition of tyrosinase is found in phenylketonuria, a Mendelian recessive trait characterized by mental deficiency and decre[illegible]
syndrome
alanine to
amount or complete absence of L-phenylalanine oxidase in the liver, a normal amount of the apoenzyme with a coenzyme missing, or a normal amount of L-phenylalanine oxidase inactivated by an inhibitor (Dalgliesh, 1955). In any event the metabolic block leads to an accumulation (in blood or urine) of phenylalanine, phenylacetylglutamine, phenylpyruvic acid, and *p*-hydroxyphenylacetic acid

A striking example of phenylketonuria is described by Jervis (1937) as "an idiot baby with blond hair and blue eyes who belonged to a family of Sicilian extraction, all the members of which, for at least three generations, were of a very dark Mediterranean race" In the Asiatic race the decrease in hair color is more obvious, and Shizume (1957) has observed, in four Japanese children, a *brown* hair color in phenylketonuria while the normal hair color of the Japanese is *black*.

Three possible hypotheses have been advanced to explain the reduction of melanin formation in the hair bulb (Dalgliesh, 1955) (1) the absence or decrease of the melanin precursor, tyrosine, (2) the absence or decrease of tyrosinase in the hair bulb, and (3) the inhibition of the tyrosine-tyrosinase reaction by phenylalanine or related abnormal aromatic metabolites which accumulate in the blood of patients with phenylketonuria The latter is the most likely since a darkening of hair color occurs in patients with phenylketonuria, maintained on synthetic diets deficient in phenylalanine (Bickel *et al.*, 1954, Woolf *et al.*, 1955, Armstrong and Tyler, 1955) Darkening of hair color is associated with the return to normal of blood—and presumably tissue—phenylalanine levels in patients maintained on the synthetic diet. Furthermore, Dancis and Balis (1955), using plant tyrosinase and Miyamoto and Fitzpatrick (1957a), using mammalian tyrosinase, have shown that phenylalanine and other aromatic metabolites present in the blood of phenylketonurics can inhibit the oxidation of tyrosine catalyzed by tyrosinase, the inhibi-

tion is competitive. Inhibition of mammalian tyrosinase by L-phenylalanine at the same concentration as in the blood of patients is about 15–30%, however, both the enzyme (tyrosinase) and the substrate (tyrosine) are present in the hair bulb in much smaller amounts than

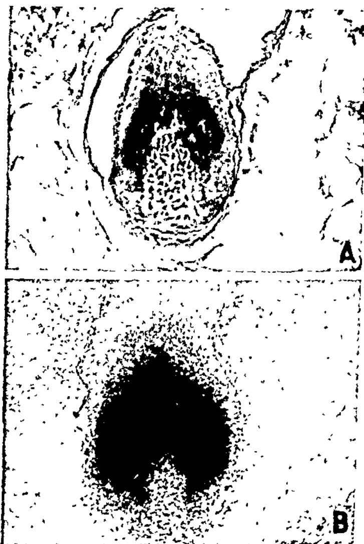


FIG 18 Normal human black hair (A) The melanocytes occupy the upper portion of the hair matrix Hematoxylin and eosin Magnification × 320 (B) Radioautographic tyrosinase method Lithium carmine Magnification × 320 The tyrosinase contained within the hair bulb melanocytes has catalyzed the oxidation of C^{14}-labeled tyrosine to C^{14}-labeled melanin The sites of radioactive melanin are represented by the dense masses of silver grains

were used in the *in vitro* studies Thus, a greater inhibition by L-phenylalanine might be expected *in vivo* The mechanism of the decreased melanin formation in phenylketonuria is summarized in Fig. 20

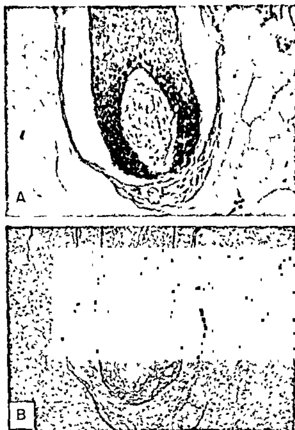


FIG 19 Human gray (with ageing) hair bulb (A) No melanocytes are visible in the upper portion of the matrix, but electron micrographs must be examined before it ... gray hair bulb Her... ...ic tyrosinase meth... ...er portion of the hair matrix Therefore, no tyrosinase activity is detectable in the human gray hair bulb

The incorporation of tyrosine into proteins of liver slices is inhibited by high concentrations of L-phenylalanine (Bickis *et al.*, 1957) Perhaps the best evidence of a tyrosine deficiency at the enzyme site is the darkening of hair color in phenylketonuric patients following oral administration of large amounts of tyrosine (Snyderman *et al.*, 1955).

By supplying an excess of tyrosine, the normal ratio of the inhibitor (phenylalanine) to metabolite (tyrosine) is restored, and normal melanin formation occurs *The tyrosine-feeding experiments of Snyderman et al. (1955) also provide the first conclusive evidence that tyrosine is the precursor of melanin in vivo.*

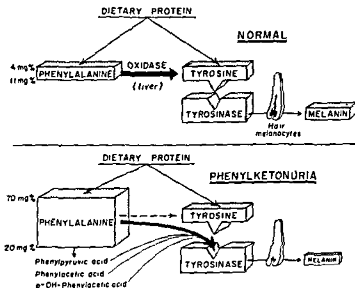


FIG 20 Mechanism of decreased melanin pigmentation of hair in phenylketonuria

2 *Noncompetitive*

Although a number of chemicals may inhibit *in vitro* melanin formation, only a few have been shown to affect hair color in animals Hydroquinone causes a reversible depigmentation in cats (Martin and Ansbacher, 1941) and rodents (Oettel, 1936), but the mechanism of its action in the tyrosine-tyrosinase reaction is not completely understood Hydroquinone inhibits the first step or the conversion of tyrosine to dopa (Denton *et al*, 1952), but nothing is known about the type of inhibition

Certain organic sulfur-containing compounds have been shown to be effective inhibitors of hair pigmentation (Dieke, 1947) Phenylthiourea, at average daily levels of 25 mg/kg, caused a graying of hair in black and brown rats but had no effect on the hair growth *α-Naphthylthiourea, a rat poison, inhibited hair growth* as well as hair pigmentation The effects of both phenylthiourea and α-naphthylthiourea are reversible since hair pigmentation and hair growth resume after withdrawal of the drugs Phenylthiourea is one of the most potent

inhibitors of the tyrosine-tyrosinase reaction, and causes a 50% inhibition at concentrations as low as 10^{-6} *M* (Lerner *et al*, 1950). Although it has been reported that the inhibition of the tyrosine-tyrosinase reaction by phenylthiourea is caused by a binding of the copper prosthetic group of tyrosinase (Lerner *et al*, 1950), this has not been proved experimentally. The inhibition of tyrosinase by phenylthiourea cannot be reversed by the addition of copper.

VI The Metabolic Pathway to Yellow Hair Pigment

Little is known about the chemistry and process of formation of yellow pigment, and many of the experimental findings are curiously anomalous, nevertheless, some definite information has been obtained from genetical work, and it is worthwhile to attempt to correlate this with the rather sparse chemical information (Wright, 1942, Russell *et al.*, 1948, Haldane, 1954). This section must be largely speculative and heuristic.

A Theories of the Nature and Formation of Pheomelanin

Pheomelanin and melanin have been considered to be derived from the same precursor, tyrosine, and each has a common metabolic pathway Both tyrosinase (Foster, 1951) and dopa oxidase (Russell, 1939, Ginsburg, 1944, Russell and Russell, 1948) activity have been detected by incubating tissue containing hair follicles in these substrates Tyrosine-2-C^{14} is incorporated *in vitro* at sites of black pigment formation (Kukita and Fitzpatrick, 1955) as well as in the pheomelanic hair follicles of man, guinea pigs, mice and in the pheomelanic feather papillae of fowls (Brunet *et al*, 1957). Thus both pheomelanic and melanic hair follicles may oxidize tyrosine and dopa in life. However, dopa oxidase activity is more closely correlated to the content of pheomelanin than that of melanin (Russell, 1939, Ginsburg, 1944; Russell and Russell, 1948) On this, Haldane (1954) has commented that "whatever dopa oxidase is doing in mouse skins, it is not oxidizing 3-4-dioxyphenylalanine, though it is very probably concerned in some phase of the production of yellow pigment from a precursor." This discrepancy may simply be the result of the abnormal conditions under which experimental techniques are carried out Tyrosine oxidase activity has not been detected in pale pheomelanic hair follicles (Foster, 1956a), but this probably reflects the insensitivity of the manometric and colorimetric tests used Until the more sensitive autoradiographic technique had been applied to a range of color forms, these negative results remained questionable

Should it prove to be the case, as seems likely to, that all colored

hair follicles show tyrosinase activity, the part that this enzyme plays in the formation of black and yellow pigment still remains to be settled Mammalian tyrosinase is thought to act specifically on tyrosine, which indicates that tyrosine itself is implicated in the formation of pigments, but it does not necessarily indicate that tyrosine is the chromogen. In melanic pigment, the deposition of pigment *in vitro* closely resembles the process of natural pigment formation, and there is at present no good reason for thinking that the natural black pigment is anything but an oxidized and polymerized product of tyrosine bound to the pigment granules This, however, is not the case with pheomelanin; when pheomelanic follicles are incubated with tyrosine or with dopa, they oxidize these substrates producing, not a yellow pigment, but an unnatural black pigment. Despite this observation, the difference between melanin and pheomelanin has been variously attributed to the degree of polymerization of the tyrosine oxidation product, to its method of attachment to the protein of the melanin granule, to its state of oxidation, or to the two pigments being the end products of different pathways of metabolism, presumably subsequent to the formation of dopa quinone These theories are largely speculative The greater solubility of pheomelanin, compared with melanin, in dilute alkalis could result from the different combination of chromogen monomers with themselves or with the protein of the pigment granule, such solubility difference might also be expected if pheomelanin were a completely different type of pigment from melanin Melanin and pheomelanin have been considered as reversible oxidation reduction products of each other Saller and Maroske (1933) considered that dark melanin was the primary oxidation product of dopa and that since hydrogen peroxide converted this pigment to a tan-colored, and then to a colorless, substance these chemical oxidation stages were represented by the series melanin-pheomelanin-albino substance. This is not possible since no oxidative activity, such as would produce even the black pigment from dopa, has been detected in white hair follicles and no enzyme capable of oxidizing dopa melanin in this way is known (Danneel, 1936, Russell and Russell, 1948). There is a view that pheomelanin represents a further oxidation product of melanin (Arnow, 1938, Nickerson, 1946) Concerning the theory that black and yellow pigments are different in that, while both are derived from tyrosine, they are formed from a different oxidation pathway, it can only be said that tyrosine, in the presence of tyrosinase, is converted to a black substance and no enzyme is known that leads to the formation of a stable yellow oxidation product

The relation of melanin and pheomelanin is on a sound genetical base The genes that lead to dilution of pigment act differently accord-

ing to whether melanin or pheomelanin is present. In all animals studied, genes at the *C*-locus (the albino series) affect pigmentation in such a way that if *C* is present, pigmentation is intense, and if *c/c* is present, pigmentation is absent If genes producing intermediate coloration are present, the ratio of pigment present in the mutant to that of the full-colored form is different in melanic and pheomelanic animals. In the guinea pig, the genes for pink-eyed dilution, *p/p*, reduce melanin, but not pheomelanin, also the genes *f/f* dilute pheomelanin, but not melanin The palomino horse has yellow body hair, it has genes that would potentiate the formation of a brown mane and tail were it not for the presence of the dominant dilution genes, *D*, this dilutes the brown melanin, giving the animal a cream-colored mane and tail, but has much less effect on the yellow pheomelanic body hair (Castle, 1954).

Black and brown melanins seem to be closely related chemically and in their mode of formation Both have the same solubility properties, and both are similarly affected by the albino series of genes and by the dilution genes The difference in color could be caused by an oxidation reduction phenomenon, by difference in polymerization of the pigment, or by the nature of attachment of the melanin to the protein granule

In summary, black and brown melanin appear to be closely related chemically, and genetical evidence indicates that their modes of formation are closely similar. Pheomelanin differs chemically from melanin and genetical evidence indicates a very distinct mode of formation for the two pigments Tyrosinase is involved in the formation of all the pigments, and tyrosine may be considered to be the chromogen for melanin While tyrosine will act as a substrate for pheomelanic hair follicles *in vitro*, the pigment formed is abnormal, and there is little indication that tyrosine is the natural chromogen of pheomelanin: the oxidation of tyrosine by pheomelanic hair follicles may be involved only indirectly in pigment formation

B *Tryptophan Metabolites and Pigment Formation*

In the formation of pheomelanin three points must be taken into account

(1) Melanin and pheomelanin can coexist in any one animal together with white Determination of the type of pigment in the coat must depend on local conditions in the follicles Different types of melanin may occur in different organs dark melanin often occurs in the iris of the eye when the hair is pheomelanic In bicolored hairs such as the agouti hair, one follicle secretes the pigments, the type of pigment secreted varying with the stage of the hair growth cycle. Systemic conditions may alter the intensity of the pigment present in any locality

(2) The activity of the genes for pheomelanin production, *e/e* in the guinea pig, or A^y in the mouse, "turn on" the production of pheomelanin in a definite way, and no intermediates between melanin and pheomelanin are formed. This clear-cut action of the genes presupposes a clear-cut switch mechanism, probably involving only one enzymatic step, which is probably present in the follicles.

(3) Pheomelanin formation must involve the activity of tyrosinase, but without necessarily implicating tyrosine as the pigment precursor.

A series of red, brown, and golden-brown pigments, derived ultimately from tryptophan, occur in the eye and integumental pigment in insects, cephalopod molluscs, and crustaceans (Butenandt *et al.*, 1954a, b ,c, d, Butenandt and Neubert, 1955; Butenandt and Beckmann, 1955; Ephrussi, 1942, Tatum and Beadle, 1940, Becker, 1941; Kikkawa, 1941, Caspari, 1949). Xanthommatin, the most common and best known of these pigments is a benzophenoxazone, derived from the oxidative condensation of two molecules of 3-hydroxykynurenine.

The body fluid of *rb/rb* mutant silkworms turns red in air, while the body fluid of the wild type turns black, probably caused by the presence of 3-hydroxykynure in the body fluid of the mutant (Inagami, 1954). In the presence of tyrosinase, solutions of dopa or 3-hydroxykynurenine produce black pigment in the mutant, a yellow solution in the wild type, and a black-brown, red-brown, or red in mixtures of the two. The chromogenic reaction depends on the conversion of dopa to dopa quinone in the presence of tyrosinase, and the nonenzymatic oxidation of 3-hydroxykynurenine to xanthommatin by the dopa quinone which was reduced back to dopa (Butenandt *et al*, 1956) Another *o*-aminophenol derived from tryptophan, 3-hydroxyanthranilic acid, is also oxidized to a pigment in the presence of dopa and tyrosinase (Butenandt, 1957a, b). This is represented in Fig 21, side chains have been omitted for simplicity although they undergo change during the reaction

When the red hair bulbs (Fig 22) of man and those of intense yellow, *e/e*, guinea pigs are incubated with dopa, they form black pigment, but when incubated with dopa and 3-hydroxykynurenine in the molar ratio of 1 to 4 for 20 hours, they produce a dark solution resembling the pheomelanic hair in color (Brunet *et al*, 1957). if the ratio of substrates is 4 to 1, no black pigment is formed within 4 hours, but after 20 hours at 37°C, black pigment is deposited Pheomelanin, thus, is formed as the result of oxidation of an *o*-aminophenol by dopa quinone, produced by the oxidative action of tyrosinase on dopa This is compatible with the fact that pheomelanic hair follicles contain tyrosinase, it also explains the pigmentary switch mechanism leading

either to melanin or pheomelanin as a result of the absence or presence of *o*-aminophenol in the follicle. The critical enzyme operating the switch could be one bringing about hydroxylation of an aromatic amine. When kynurenine is substituted for 3-hydroxykynurenine in the above experiments, its effect on the deposition of black pigment is very slight. The yellowish-brown pigments formed by the oxidative condensation of *o*-aminophenols are soluble in dilute alkalis, as in pheomelanin. Aromatic amines, but not amino acids, combine with dopa quinone and form pigments (Glassman, 1957). Pheomelanin might be such a mixed pigment.

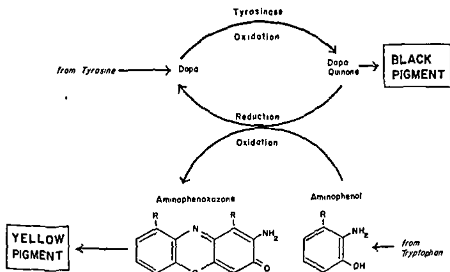


FIG 21. Tyrosinase system and the oxidation of aminophenyl compounds (After Butenandt *et al*)

Pheomelanic hair is fluorescent when illuminated with light of wavelength 3600 A and emits a light varying from dull orange to bright yellow (Brunet *et al*, 1957) Yellow hair, the pheomelanic banding of agouti hair of mice, guinea pigs, and golden hamsters and human red hair show this effect The fluorescence of a mixture of 3-hydroxykynurenine and dopa, in which hair follicles had been incubated, was a dark orange color like that of an alkaline extract of pheomelanic hair.

No systematic search for tryptophan metabolites has been made in skin and hair, but it is interesting that Rebell *et al.* (1956) isolated from the yellowish hair of albino rats an unknown substance (their Fl spot) which is now known to be kynurenine (Rebell, 1957).

Finally, a separate metabolic pathway for melanin and pheomelanin

is suggested by the inhibitory effect of chloroquine, 7-chloro-4 (4-diethyl-amino-1-methylbutylamino) quinoline diphosphate, on the formation of pheomelanic hair pigment, but not human black or brown hair pigment. Loss of hair color following administration of chloroquine was first reported by Alving and Eichelberger (1948). Fitzpatrick and Saunders (1957) have observed twelve red-, light brown- and yellow-haired pa-

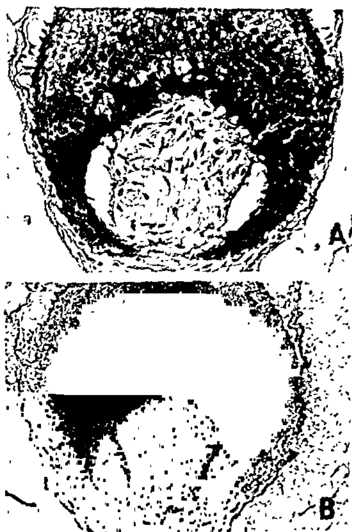


Fig. 22 Human red scalp hair (A) Melanocytes occupy the upper portion of the hair matrix Hematoxylin and eosin. Magnification × 320 (B) Radio-autographic *tyrosinase method Lithium carmine* Magnification × 320 The intense concentration of silver grains indicates marked tyrosinase activity in the melanocytes of the human red hair bulb.

tients receiving chloroquine for lupus erythematosus and polymorphous light eruptions who have shown complete loss of hair pigment while ingesting the drug. The pigment returned following cessation of therapy with chloroquine. However, in one of the patients with red hair, certain portions of the hair which had become white developed a brown color after the drug was stopped. This suggests an irreversible inhibition of the metabolic pathway of red pigment. One Negro patient receiving large doses of chloroquine for 3 years has not shown loss of pigmentation. There has been no depigmentation in Japanese receiving chloroquine The mechanism of chloroquine inhibition of human red hair color is not known but further investigation is in progress. In a recent study we have observed depigmentation of the feather papillae in Rhode Island Red chick embryos in which chloroquine was injected into the yolk sac on the fifth day of development. Acetylkynurenine was isolated from these depigmented papillae but not from the normally pigmented feather papillae of Rhode Island Red chicks.

VII. Appendix

1. Use tissue 1 mm in thickness
2. Incubate tissue slices in following mixture
   (a) C^{14}-labeled tyrosine, 0 3 microcuries (DL-tyrosine-2-C^{14})
   (b) O 1 *M* phosphate buffer, pH 6 8, 1 cc
   (c) Penicillin, 3000 units
   Dissolve C^{14}-tyrosine and penicillin in buffer then add *1 cc* buffer to the solution for incubation
   For control Use a solution containing (a), (b), (c), then add 1 cc of 0 01 *M* diethyldithiocarbamate in phosphate buffer.
3. Incubate for 24 hours in metabolic shaker
4. Fix in 10% formalin for 1 hour
5. Wash in running water overnight.
6. Fix again in 10% formalin for 24 hours
7. Imbed in paraffin
8. Cut tissue at 4 μ
9. Float tissue ribbons in distilled water at 42°C
10. Transfer flattened tissue ribbons to distilled water of room temperature.
11. Turn off bright light, use Wratten No 2 red filter for the following steps
12. Scoop tissue ribbon onto the emulsion side of track film NTB_3 swiftly.
13. Dry quickly with fan (about 10 minutes)
14. Deparaffinize with two changes of cold xylene, 10 minutes in each solution
15. Dry again with fan, until no wrinkles are visible (about 15 minutes)
16. Put finished slides in light-tight box in a desiccator in cold room for exposure (*2 weeks*)
17. Develop the film with full strength Kodak DK 19 for 10 minutes at 20°C
18. Wash in distilled water for 1 minute
19. Fix in Kodak acid fixer (full strength) for 5 to 10 minutes, or until film clears at 20°C
20. Wash in running water for 30 minutes

21. Rinse in distilled water.
22. Stain in cold lithium carmine
23. Run through cold acid alcohol, 70% and 95% alcohol, saturate picric acid in 100% alcohol, 100% alcohol xylene.
24. Bring slides to room temperature and mount in permount.

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CHAPTER 14

Nutritional Factors Influencing Hair and Wool Growth

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I. Introduction

That diet influences the coat of an animal has long been recognized, but progress toward understanding the effects of poor nutrition on hair growth has been slow. One reason for this is perhaps the difficulty of defining precisely what is meant by the lack of "bloom" or dry and disordered appearance of the coat of an animal in poor condition. Another probable reason is that the hair growth cycles of different animals have only fairly recently been described A considerable amount of work has, however, been carried out on the influence of nutrition on wool growth in sheep in which most follicles are active at any one time (Fraser, 1934, Krishnan, 1939, Marston, 1946, 1955). A poor grazing season produces fleeces of low weight, conversely, abundant food stimulates wool production The maximum quantity of wool grown by a sheep, however, is determined genetically. Although nutrition is important in providing the means whereby a sheep can express its genetic

potentialities for wool production, no amount of extra feeding can make it exceed the genetic maximum If a sheep responds to an addition to its diet, then the diet must have been deficient in the substance added (Fraser, 1934). It could be, however, that under normal conditions it is rare for a sheep to produce wool at the maximum rate of which it is capable (Marston, 1955).

Van Koetsveld (1954) reviewed work on the influence of feeding on the coat of animals. Flesch (1954), in his brief review of the effect of diet on hair growth, states that impaired growth or loss of hair is a common and early nonspecific response to many nutritional deficiencies in animals In human beings a very poor diet is needed to cause loss of hair, probably because a smaller fraction of the total available protein is diverted to hair formation.

II. The Influence of Diet on the Rate of Fiber Production

The rate of wool growth in sheep is measured as the weight of clean, dry wool produced by a certain area of skin in a certain time. The area is tattooed in the skin and initially clipped, the weight of wool grown in a pre-experimental period can then be compared with the weight grown in the same area while the sheep is receiving different diets. In this way it was found that wool production in the Merino breed varied over a range of 400% under different grazing conditions (Marston, 1955)

Studies of the rate of hair growth in animals other than sheep take advantage of the fact that plucking the hairs from quiescent follicles initiates regrowth of hair. Loewenthal and Montagna (1955) found that if the diet of mice is reduced to one-third of the normal diet and hairs are plucked from the follicles on the day the animals are placed on the reduced diet, or 3 days before, the follicles remain quiescent for as long as the diet remains reduced. When the hairs are plucked 5 days before the animals are placed on the reduced diet the follicles show a considerable delay in the completion of the cycle of hair growth If, however, the hairs are plucked 10 days before, the follicles complete their cycle of growth in a normal time. When hairs are plucked at the time the animals are placed on a reduced diet, the follicles remain quiescent for as long as 2 months, but they become active on the very day the animals are given food *ad lib*

Let us now consider the importance of different dietary factors.

A. *Protein*

Although the rate of wool growth is influenced by the amount of protein in the diet, the protein requirements of a sheep are not par-

ticularly high (Fraser and Roberts, 1932). Fleece weights can be increased by giving supplements of protein to grazing sheep, but addition of protein beyond a certain limit has no further effect (Drummond and Bassett, 1952, Slen and Whiting, 1952). Attempts to make use of urea as a source of nitrogen have been unsuccessful in increasing the growth of wool, even when sulfur was added (Slen and Whiting, 1955).

The ultimate nutritional condition determining the rate of wool growth seems to be the concentration of essential amino acids in the tissue fluids around the follicle, and whereas the supply of amino acids to the animal is determined by the amount and kind of protein in the diet (supplemented in sheep by proteins synthesized by the microflora of the rumen), the quota that eventually becomes available for wool production is determined by the nutritional state of the animal (Marston, 1955). When the energy requirements of the animal are not completely met by carbohydrate and fat, protein is used for fuel and there is less for other purposes. The synthetic potential of the proliferating cells of the follicle bulb is high relative to that of other tissues, a fasting sheep is believed to continue growing wool virtually at the expense of its other tissues. Although in these circumstances the rate of wool production is considerably reduced, it is not much less than when the animal is provided with just sufficient fodder to maintain itself in energy equilibrium, and the call on protein in the fodder to serve as fuel is considerable. The effect of adding protein to the diet seems to lessen the competition of other tissues

B. *The Amino Acid Cystine*

Since keratin is not a homogeneous protein, there are discrepancies between the many different analyses that have been published. The results of three analyses are shown in Table I. However, a consistent feature of all analyses is the relatively high content of the sulfur-containing amino acid, cystine, the amount of which approaches 10% This high cystine content of keratin and the low cystine content of grass (1–2%) led some to believe that the capacity of sheep to produce wool might be determined by the capacity of the plants to produce cystine One of the assumptions of this hypothesis is that the sheep could not synthesize cystine. Studies with radioisotopic techniques show that sheep can synthesize cystine from sulfate, but not so easily from sulfur (Hale and Garrigus, 1953). Marston (1955) considered that because the cystine and potential cystine content of grass total only about one-third that of wool, only about 30% of the other amino acids can be used for wool production. This percentage, however, is rarely approached and seldom more than 10% of the plant protein is used in wool production

The cystine of wool and hair has been of considerable interest, pa ticularly in determining whether or not additions of cystine to the di are beneficial (Fraser, 1934). Subcutaneous injections of cystine increase wool growth for a period of 3 weeks afterward Du Toit *et al.* (1935 found that a supplement of cystine did not produce more wool, bu

TABLE I
AMINO ACID ANALYSES OF WOOL BY VARIOUS AUTHORS[a]

	Nitrogen as % total nitrogen of wool		
Amino acid	Simmonds (1954)	Corfield and Robson (1955)	Graham, Waitkoff, and Hier (1949)
Alanine	3 51	4 12	—
Amide N	7 46[b]	6 73	—
Arginine	20 30	19 1	21 1
Aspartic acid	4 24	4 38	4 7
Cystine	7 93(4 95)	7 30	9 9
Glutamic acid	8 58	8 48	9 2
Glycine	5 80	6 49	—
Histidine	1 46	1 91	1 8
Isoleucine	1 97	2 44	3 0
Leucine	4 90	5 85	5 3
Lysine	3 25	3 92	3 9
Methionine	0 39	0 32	0 4
Phenylalanine	1 75	2 12[c]	2 1
Proline	5 33	5 05	6 1
Serine	7 25	8 66[c]	—
Threonine	4 61	5 12[c]	4 8
Tryptophan	1 73	0 82	—
Tyrosine	2 97	2 62	2 7
Valine	3 57	4 16	4 2
Unknown (1)	1 18	—	—
Unknown (2)	0 71	—	—
	98 89	99 39	79 2

[a] From Corfield and Robson (1955)
[b] Uncorrected for decomposition of serine and threonine during hydrolysis
[c] Corrected for loss during hydrolysis

Marston (1955) found that a cystine supplement added to a low protein diet did The lack of cystine retarded hair growth in rats, and the cystine content of the hair was reduced (Smuts *et al*, 1932), but feeding excess cystine did not alter the cystine content. The presence of either cystine or methionine in the diet is essential for hair growth in rats and when rats were fed on a diet containing little sulfur the cystine content of the hair was reduced (Block, 1949) The fur of rabbits was found to undergo seasonal variations in the sulfur content, but no differences occurred

in sulfur content when rabbits were fed extra cystine (Barritt *et al.*, 1930). Reports indicate that the addition of cystine to the diet led to an increase in the weight of the fur of rabbits (Deravlev, 1935), and that the feeding of a hydrolyzate of human hair to rats produces cystine contents three times as high as those of control animals (Takahashi and Shirahama, 1940). The cystine content of hair varies with the age of an animal (Van Koetsveld, 1954).

C. *Substitutes for Cystine*

Naturally occurring cystine is L-cystine. The rat can oxidize *i*-cystine (presumably DL), but its value for the building of tissue was only about half that of L-cystine (Lawrie, 1932). Du Vigneaud *et al.* (1932) found that D-cystine could not be used for growth in lieu of L-cystine. Dyer and du Vigneaud (1936) found that glutathione could be used as a substitute for cystine Methionine is the most efficient substitute for cystine in several species (Krishnan, 1939), and methionine can be converted to cystine in the body (du Vigneaud *et al*, 1944). Neither sulfur nor sulfate increased wool growth (Krishnan, 1939), although sulfate can be converted into an organic form by sheep

Lambs are said to utilize sulfur and to retain more nitrogen than lambs receiving no additions of sulfur (Starks *et al*, 1953). The additional sulfur in the diet did not alter the sulfur and nitrogen contents of their wool. Loftgreen *et al* (1953), on the other hand, found that the addition of 0 2% of sodium sulfate to a diet in which urea provided 40% of the nitrogen was without effect on nitrogen retention and wool growth. Additions of methionine increased nitrogen utilization in a diet containing urea (Gallup *et al*, 1952) Small amounts of radioactive cystine have been recovered in the hair of a rat following injection of labeled sulfate (Dziewiatkowski, 1954) Inorganic sulfur would, therefore, seem to be all that is essential for ruminants although other animals appear to need amino acids containing sulfur.

D *Carbohydrate*

When sheep were given a starch supplement, there was a significant increase in the weight of the body and that of the fleece Carbohydrate in the diet converts a negative nitrogen balance into a positive one (Krishnan, 1939). Pierce (1951) found that the addition of starch made urea utilizable The explanation that carbohydrate frees protein for wool production which would otherwise have been used to provide energy is probably too simple. Carbohydrate is needed in order that amino acids can be utilized, and unless carbohydrate is fed at the same time as protein, the latter is not so readily utilized and nitrogen excretion increases (Munro, 1957). Carbohydrate is needed for mitosis to take

place (Bullough, 1952), and the importance for hair growth of glycogen in the follicle has been amply demonstrated.

E. Fat

It is unusual for sheep to have a definite adipose layer, clumps of fat cells are sparsely distributed throughout the skin, and the amount of fat varies over the year. In rats, during the active phase of hair growth, there is a considerable thickening of the adipose layer (Durward and Rudall, 1949) Borodach and Montagna (1956) found, however, that this increase in volume of the fat is brought about largely by the downgrowth of the follicle bulbs into the adipose layer, chemical analysis showed that except for slight increases in the early stages of follicle growth the fat content of the skin remained unaltered during the cycles of hair growth. This suggests that there is no specific association of fat with hair growth.

Marked changes occur in the skin of dogs on a low fat diet (Hansen et al, 1954). These included shrinkage and fragmentation of the hair shafts, conditions became normal when fat was added to the diet It has been suggested that fat may act through the secretion of the sebaceous gland (Van Koetsveld, 1954), i e, the change in the condition of the hair caused by fat or grease is probably due to its deposition on the surface of the fiber. Horses are fed oats with a high oil content in order to maintain hair gloss.

F. Specific Deficiencies

Only those deficiencies that seem to have a specific effect on hair growth are emphasized here, because any deficiency that causes a reduction in food consumption will affect hair growth.

1 *Vitamins*

The following deficiencies are said to cause impaired hair growth. vitamin A, riboflavin, biotin, inositol, pantothenic acid, pyridoxine, and vitamin E (Flesch, 1954)

Vitamin A is said to prevent the keratinization of epithelia that are not normally keratinized Deficiency of vitamin A does not seem to have any effect on wool growth (Pierce, 1945) Deficiency in the mouse inhibited spontaneous growth of hair, but not growth initiated by plucking (Montagna, 1956). The local depilatory action of vitamin A in animals is probably not specific (Flesch, 1953)

Wool production is not complicated by deficiencies of the vitamin B complex, the sheep's supply of these vitamins being assured by the activity of the microflora of the gut (McElroy and Goss, 1940, Marston,

1946). The vitamins of the B complex are, however, important in other animals, and vitamin B, taken over long periods, was found to restore human hair pigmentation (Lanczos, 1941). During biotin deficiency mice maintained the cycles of hair growth, but the hairs were shed at the completion of growth (Rauch, 1952). Riboflavin deficiency caused a loss of hair in rats (Sullivan and Nicholls, 1941), deficiency of pantothenic acid causes poor hair growth and loss of pigment. Deficiency of riboflavin and pantothenic acid in Negro children not only caused depigmentation, but made their hair grow straight (Hughes, 1946). This deficiency seems to be associated with the utilization of copper. The skin of rats deficient in pantothenic acid may contain as much as five times the amount of copper in normal skin, i.e., the copper cannot be utilized and accumulates (Hundley and Ing, 1951).

2 *Minerals*

Deficiency of copper apart from causing loss of pigment in hair and wool causes sheep to produce wool that lacks waviness or crimp (Marston, 1946). Additions of copper to the diet or applications of copper ions to the skin surface restores the growth of normal crimpy wool (Marston, 1955). An excess of molybdenum in pastures induces copper deficiency in sheep by fixing copper in a form that cannot be utilized (Dick, 1954, Wynne and McClymont, 1956)

A deficiency of zinc caused rats and mice to lose hair (Day, 1942; Day and McCollum, 1940, Follis *et al.*, 1941), and iron deficiency caused hairlessness in rats (Cunningham, 1932). Cobalt deficiency has a marked effect on the condition of ruminants (Cuthbertson, 1954), and although cobalt-deficient sheep produce less wool with weak fibers (Keener *et al*, 1950), there is apparently no specific effect on wool growth Phosphorus deficiency is a major agricultural problem in many parts of the world. Phosphorus deficiency reduces body weight and fleece weight (Duerden *et al.*, 1932). The deficiency does not affect wool growth if protein consumption is kept constant. Changes in calcium intake caused no differences in wool growth, and iodine deficiency has no effect However, in thyroid deficiency caused by low iodine intake, there was loss of hair and wool (Cuthbertson, 1954) This indicates the importance of thyrosine in controlling hair growth

III The Influence of Diet on the Nature of Wool and Hair Produced

A reduction in clean fleece weight is associated with a reduction in the length and diameter of individual wool fibers (Fraser, 1934, Coop, 1954, Ryder, 1956a). It has been suggested that change in fiber diameter

is mainly due to change in the width of the medulla (Krishnan, 1939). But Coop (1954) and Ryder (1956a) found that it was caused by changes in the thickness of both cortex and medulla.

The differences in the diameter of a fiber at different levels of nutrition seems to be due to the relative size of the bulb (Marston, 1955) The size of the keratinized cells of fibers remains practically unaltered whatever the size of the fiber, although the cells of coarse fibers may be larger than those of fine fibers (Appleyard, 1957). Change in the length of fibers grown depends on the rate of cell division, whereas change in diameter depends on the number of the cells.

A. *The Mechanism of Hair and Wool Loss*

Hair and wool are thought to be shed under poor nutritional conditions, but a factor that complicates such observations is the hereditary tendency of many sheep to shed or molt seasonally (Ryder, 1956a, 1957a) The fibers form "brush ends" and fall out, after a short resting stage a new fiber grows. Molting, which takes place in spring, probably contributes to the "winter break" in fleeces. This is a level of the fleece at which the fibers are thinner, probably as a result of poor winter feed. Some fibers are so thin that they break, but others may have stopped growing Fiber thinning and fiber shedding by brush formation ar two distinct phenomena, one is due to poor nutrition, and the other to completion of a growth cycle

In an attempt to determine whether or not a poor diet would induce brush formation, two sheep were placed on a diet similar to one that they often receive in winter (Ryder, 1956a) After 5 weeks they lost one-quarter of their body weight, whereas two control sheep gained in weight Little fiber shedding occurred Fiber shedding took place during the following winter, even when the sheep were fed well Fiber shedding by brush formation is apparently seasonal and not caused by nutrition Molting in cattle is associated with changes in the number of hours of daylight and probably with endocrine secretions (Yeates, 1954) Good feeding apparently hastens regrowth of fibers After shedding in sheep, a significantly greater number of fibers regrew in a group of animals receiving supplementary feeding (Ryder, 1955a).

Malnutrition reduces the breaking strength per unit cross-sectional area and makes wool fibers thinner (Ventner, 1953). The average values of tensile strength of fibers from sheep corresponding to periods of sufficient, poor, and again adequate feeding are 1.09, 0.75, and 1.21 $\times 10^6$ gm/cm^2

IV. The Influence of Diet During the Period of Follicle Development on the Ultimate Capacity of Animals to Produce Hair and Wool

A sheep stunted by malnutrition may not be able to express its full hereditary powers of wool production (Marston, 1955). The quality of food available during fetal and early life could impose a permanent limitation on the subsequent wool-producing capacity by reducing the number of follicles developed However, although poor nutrition retarded fleece development. It had no subsequent effect on wool production (Henderson, 1953).

Poor nutrition does not prevent newborn rats from growing hair (Jackson, 1932), although hair formation is considerably delayed (Butcher and Richards, 1939).

In sheep it is important to distinguish between the primary follicles, which are all formed before birth (characterized by the possession of sweat glands and arrectores pilorum muscles) and the secondary follicles which continue to be formed after birth (Ryder, 1957a). One can then investigate the influence of nutrition on the development of wool follicles by determining the ratio of secondary to primary follicles in skin samples taken at different ages from lambs whose mothers are on different planes of nutrition

There are two conflicting views on the effect of nutrition on the postnatal development of follicles One states that the number of follicles a sheep will form cannot be influenced by feeding (Hugo, 1953, 1954). The other claims that sheep, kept on a poor nutrition from 6 weeks until 3½ years of age, have, at the end of the period, a smaller total number of follicles than control animals (Marston, 1955).

Ryder (1955a) took skin samples at birth and at weaning from two groups of Cheviot lambs whose mothers were on supplemented and unsupplemented diets during pregnancy and lactation No significant difference was found in the secondary to primary follicle (S/P) ratio between the groups at either sampling time, which indicated that the diet of the mothers had not influenced the number of secondary follicles formed. However, the S/P ratio at birth and at weaning, were both correlated with body weight at birth, illustrating the importance of fetal environment for secondary follicle formation and final S/P ratio. The correlation between S/P ratio at birth and birth weight supports Schinckel's hypothesis (1953) that the amount of food available to a fetus can make a difference in the number of secondary follicles formed The lack of significant difference in S/P ratio between the groups at weaning, when there was a significant difference in body weight, supports Hugo's hypothesis (1953, 1954) that the number of follicles a

lamb produces cannot be influenced by feeding. Schinckel, however, was considering the amount of food available to the fetus, and Hugo the plane of nutrition of the mother. Since Hugo showed that body weight at birth had been affected by the diet of the mother it would seem that the development of follicles could be hastened by giving the mother large amounts of food, but this would probably be impossible in practice. The prenatal period of secondary follicle formation is more sensitive to nutritional change than the postnatal period (Wildman, 1957a).

Schinckel (1955a) clearly distinguished between the initiation of follicle development and the growth of fibers, which he termed follicle maturation Follicle maturation in the Merino follows a sigmoid curve when judged from S/P ratio figures in which only those secondary follicles containing fibers are included, the most rapid rate of maturation is during the second week of life. All, or many, of the secondary follicles are initiated before birth; the adult S/P ratio is determined prenatally and is not influenced appreciably postnatally (Short, 1955, Ryder, 1955a). In British breeds many of the follicles are initiated after birth (Ryder, 1957a) Early postnatal nourishment might permanently reduce the number of mature secondary follicles in the adult fleece, regardless of the effects on follicle initiation (Short, 1955).

The maturing of follicles in sheep under the same conditions shows a correlation between body weight and S/P ratio at birth, genetic factors contribute to variations in adult S/P ratio (Wildman, 1957b, Schinckel, 1955b) There is a positive correlation between gain in S/P ratio and gain in body weight from birth to 28 days The bigger the lamb at birth the more likely is its coat to be advanced in development, nutritional factors (milk supply) from birth to 28 days of age affect the rate of development of secondary follicles and determine whether the genetic maximum S/P ratio for an individual lamb is attained relatively early, late, or not at all in the period between birth and weaning Thus, it seems that nutrition cannot cause large differences in the number of follicles formed beyond the limits imposed by genetic factors

V. The Nutrition of the Follicle

A. *Blood Supply*

The blood vessels associated with the follicles are densest when the hairs are actively growing (Haddow and Rudall, 1945, Durward and Rudall, 1949) There is no association of vessels with follicles in mouse skin before birth (Hardy, 1952), and vessels in the fetal lamb do not become associated with the follicles until wool growth commences (Ryder, 1956c) The rate of wool growth in sheep kept on a constant diet was correlated with atmospheric temperature (*Ferguson et al.*,

1949). High temperatures may influence wool growth by causing vasodilation and a consequent increased flow of nutrients to the follicles. Vasodilation caused by unilateral sympathectomy increased wool growth on the side of the body in which the nerves had been cut (Ferguson, 1949) *There seems to be an association between wool production* and the temperature of the surroundings in sheep kept on a constant diet and a constant period of electric light over 24 hours (Wildman, 1957a). It is not certain, however, that environmental temperature has a direct effect on wool growth (Coop, 1954). Apart from the direct effect of temperature on blood flow, one must also consider the possibility that in cold conditions a greater amount of food will be used in keeping an animal warm, thus leaving less for wool growth.

Our understanding of the vascularization has been largely based on that of human skin (Spalteholz, 1927). A distinction must be made between the supply to the whole skin (the fleece) and the supply to the follicle. The differences in wool production by different regions of the fleece and the differences between the fine wool on the shoulders and the coarse wool of the hinder parts have prompted the suggestion that there are great differences in nutrient supply to different regions of the skin (Henderson, 1953). The skin is supplied by numerous small arteries, which are fairly evenly distributed over the body (Ryder, 1955b). The density of these vessels in the dermal network does not vary greatly in different parts of the skin. However, the density of vessels is greater in the lamb than the adult, the decrease is probably due to skin expansion. There is also a greater density of follicles per square centimeter of skin area in the lamb than in the adult. Therefore, it seemed that the density of follicles in the skin might be associated with the density of the dermal network of blood vessels. Although there is much variation in follicle density between different breeds of sheep (Carter, 1955, Ryder, 1957a), the many skins of different breeds examined *showed no noticeable differences in the density of the blood* vessel net. Thus, no anatomical differences were found which would account for differences in fleece growth, either over the body, or between breeds.

With the cyanol staining method of Ryder (1953), apart from the dermal network, two more horizontal nets can be seen in the skin, the middermal vessels and the subepidermal vessels. The wool follicles are supplied from either the dermal or middermal vessels, deeper follicles are not necessarily supplied from the lowest level.

The immediate supply to the follicle consists of two parts (Ryder, 1956c) Capillaries enter the papilla beneath the growing point of the fiber, and there is a basketlike network of capillary vessels surrounding

the lower third of the follicle (Fig. 1). This extends from the base of the follicle to about the level at which the fiber is keratinized. The supply and drainage vessels of the papilla are usually connected with the vessels of the surrounding net. There is a straight vertical vessel in the center of the papilla, suggesting that the blood rises to the peak first, after which it drains down the outside near the surface of the papilla. The larger the papilla, the more vessels it contains, this is



Fig. 1 Dense capillary network surrounding larger (primary) follicle in skin from advanced fetus of Border Leicester breed of sheep Magnification × 170. (From Ryder, 1956c)

interesting because there is a relation between papilla volume and fiber diameter (Burns and Clarkson, 1950) Thus, variations in fiber diameter seem to be associated with the number of vessels in the papilla.

In small follicles the surrounding vessel network has no particular arrangement or pattern In general, the larger the follicle the denser and more elaborate is the net The vessels are denser on one side of the follicle immediately above the bulb than anywhere else. This density is usually brought about by a horizontal arrangement of the vessels, so that they are frequently horizontal on the dense side and vertical on the other This pattern seems to consist of a vertical supply vessel on one

side which gives off obliquely vertical branches. These branches wrap around the follicle and become horizontally arranged on the other side The vertical supply vessel often continues into the papilla, and the horizontal vessels often run at right angles into a vertical drainage vessel, probably originating in the papilla. This concentration of vessels on one side of the follicle is interesting because it suggests that one-half of the growing fiber has a better blood supply than the other. Efforts to associate it with the bilateral nature of the fiber (thought to be connected with the crimp of wool) have failed because the concentration lies on the side opposite to that on which the keratinization of the fiber commences

Possible differences were sought in the blood supply of the follicle that would explain differences in fiber growth The only difference found was the greater number of vessels associated with follicles producing coarser fibers. However, a large follicle has an extensive blood supply because it is large, and it is not the extensive blood supply that makes it large

The vessels of the papilla are undoubtedly the source of nourishment for the cell division taking place around the papilla. Gradients are more likely in the follicle tissue than in the blood stream as implied by King and Nicholls (1933); substances leave blood vessels at points nearest to the sites in which they are required

The capillary net around the lower part of the follicle provides a possible source for substances needed at later stages in fiber formation. King and Nicholls (1933) thought that it was impossible for substances to diffuse through the follicle wall. The suprabulbar region, in which radioactivity appears soon after an injection of labeled cystine, is nearer to this source than it is to the capillaries of the papilla (Ryder, 1957b)

A possible additional or alternative function of the surrounding net is the removal of substances from the outer sheath (Durward and Rudall, 1949) Glycogen stored there could be removed and passed into the papilla as glucose, but this is not always supported by the pattern of the net.

That the vessels of the follicle are important for hair and wool growth is shown by their reduction in number when the fiber forms a brush prior to molting (Ryder, 1956c, Durward and Rudall, 1949) The reduction of vessels accompanies rather than precedes the shedding process, fiber shedding, then, is not caused by the reduction of vessels

The vibrissal follicles of sheep do not have a blood sinus divided into upper and lower halves (Ryder, 1956b) as it is in the rat and mouse (Davidson and Hardy, 1952, Melaragno and Montagna, 1953). The follicle has a surrounding network (Scott, 1955), some of the vessels

being small arteries (Ryder, 1956b). The assumption that the sinus blood is associated with hair growth (Hardy, 1951) is not necessarily true.

There is evidence that hormones control hair and wool growth; seasonal variations in wool production are thought to be brought about by changes in the length of daylight acting through the endocrine system. Certain hormones cause either constriction or dilation of vessels (Chambers and Zweifach, 1944), and the administration of thyroxine to rats causes an increase in capillary fragility as well as earlier eruption of hair (Kozam, 1952). The administration of L-thyroxine increased wool production (Hart, 1954) Thus, it is possible that at least part of the seasonal change in wool production is brought about through the blood stream by the direct dilating or constricting effect of hormones on blood vessels.

B. Sulfur

Samples of skin of mice taken from 1 to 8 hours after the injection of cystine labeled with sulfur-35 showed appreciable activity in the keratogenous zone after one hour, but the follicle bulb was only slightly radioactive (Bern, 1954; Bern *et al.*, 1955). This suggests that the cystine (or a derivative) is taken up by the keratogenous zone directly and does not pass up from the bulb. Bélanger (1956) noted that labeled methionine and cystine are also taken up by the hair follicles of the rat In the skin of mice there was an intense localization in the keratogenous zone of active follicles, the distribution remaining the same from 1 to 6 hours after the injection of labeled cystine, although the quantity increased progressively with time (Harkness and Bern, 1957). Follicles in catagen displayed a region of activity at the base of the hair shaft, but follicles in telogen showed no radioactivity. After 2 days the bulk of the activity was in the hair shaft above the keratogenous zone, and it takes

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FIGS 2–5 Autoradiographic preparations made with Kodak A R 50 film of thick frozen sections from the snout of mice killed at different times after injection of cystine labeled with sulfur-35 (Figs 3 and 4 from Ryder, 1957b)

FIG 2 Within 2 minutes there is radioactivity in the suprabulbar region immediately above the bulb Magnification. × 76

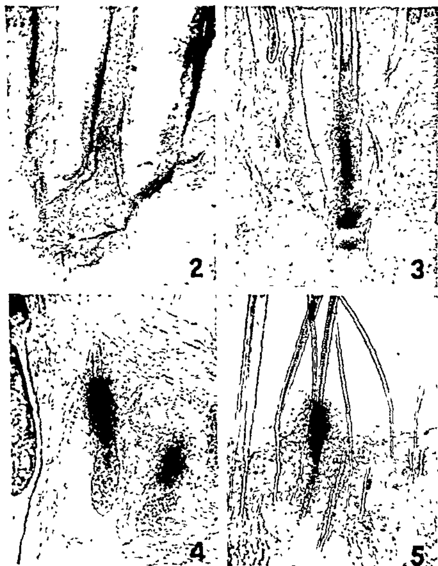
FIG 3 After 20 minutes the activity has extended some distance into the fibrillary region, but has not yet reached the prekeratinization (keratogenous) region Magnification × 75

FIG 4 After 3 hours there is a definite peak of activity in the lower part of the fiber Magnification × 112

FIG 5 The peak of activity in the fiber reaches the skin surface in about 48 hours Magnification × 66.

6 days for the activity to appear above the skin surface. After 16 days the activity in the follicle decreased virtually to zero

In order to find out the time taken for the active compound to diffuse from the blood stream into the follicle, Ryder (1956d) killed mice 6 minutes after an intraperitoneal injection of labeled cystine and found moderate activity in the follicles That passage around the body is extremely rapid can be demonstrated by injecting the tail vein of mice with



India ink; the entire animal becomes black immediately (Ryder, 1955b). It takes almost 10 minutes for a mouse to become blue after an intraperitoneal injection of chlorazol sky blue (Ryder, 1957b). Intraperitoneal injections are slower although cystine might not be retarded as much as a large dye molecule. Cystine was therefore injected with the dye via the tail vein in order to be sure that the vessel had been entered. Appreciable activity was then detected in the vibrissal follicles of a mouse killed only 2 minutes after injection (Fig. 2), and there was even slight activity in the follicles of a mouse killed only 30 seconds after injection. The time taken for cystine or derivative (possibly cysteine) to diffuse into the follicle from the blood stream must therefore be only a few seconds

Edwards (1954) applied methionine labeled with sulfur-35 to the skin of guinea pigs and found that the sulfur-35 was incorporated in the hair as cystine. The sulfur-35 content of the hair was similar whether the specimen was from the area of application or remote from it. It would thus seem that the active compound reached the follicles in the blood stream. The active compound enters the follicle quickly, and it appears first at a level immediately above the bulb (Fig. 2) Labeled cystine does not form a dense autoradiograph in the bulb One hour after an injection of glucose labeled with carbon-14, the densest autoradiograph is from the bulb (Fig. 12).

Activity did not begin to extend into the prekeratinization region until about 2 hours after injection (Ryder, 1956d) The amount of activity in the follicle increases during the first few hours after injection, and a peak is formed after about 3 hours in the lower part of the fiber (Fig 4). This peak was in the keratinized part of the fiber within 24 hours of injection and reached the skin surface in 48 hours (Ryder, 1957b) (Fig 5). The difference found in different animals (Harkness and Bern, 1957) may be explained by different growth rates The peak of activity in that part of the fiber grown soon after injection, is seen clearly in Fig 6, in the vibrissae of a mouse killed 3 weeks after injection This shows the gradual decrease of activity in the fiber with a small amount still entering the follicle even after 3 weeks

In a quantitative study of the uptake of labeled cystine by the lamb (Ryder, 1957b), the number of grains per unit area were counted in each region of the follicle (Fig 7) and the results are shown in Fig 8 The histograms show the grain density in different regions of the wool follicle at different times after injection In the first sample taken 30 minutes after injection the peak of activity was in the suprabulbar region, and there was less activity in the bulb below and in the lower part of the fibrillary region above There was more activity in the

follicle after 8 hours; the peak had moved up into the fibrillary region and there was a lot of activity in the prekeratinization region, whereas the activity in the lower part of the follicle was beginning to decrease. What is interesting here is that there was an increase in activity between the fibrillary region after 8 hours and the fully keratinized region after 24 hours. This corresponds to the enrichment of sulfur on keratini-

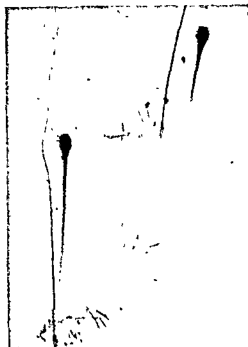


Fig 6 Autoradiographic preparation made with x-ray film of two vibrissae from a mouse killed 3 weeks after an injection of labeled cystine When this photomicrograph was taken the x-ray film was resuperimposed on the thick (frozen) sections with the image displaced to the right of the fiber A peak of activity can be seen in that part of the fiber grown soon after injection Magnification × 5

zation (Rothman, 1954). The lack of activity in the prekeratinization and upper part of the fibrillary regions in Figs 2 and 3 after 2 and 20 minutes in the mouse, and in Fig 8 after 30 minutes in the lamb, suggests that although sulfur does enter above the bulb it does not enter at such a high level One has, therefore, to fall back on the explanation that the enrichment of sulfur on keratinization is a concentration effect probably partly due to the removal of other substances.

The occurrence of a peak of activity in the follicle soon after injec-

tion suggests that the bulk of cystine injected goes immediately to the follicle. In other words there is a rapid turnover of cystine in the body, much of the compound apparently going into hair and wool. This is in accord with the view that metabolic proteins have a high rate of

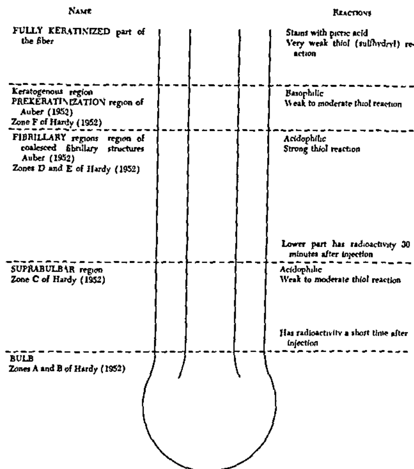


FIG 7 Diagram showing the terminology of the different regions of the follicle used by Ryder (1957b) in describing the uptake of radioactivity after injection of cystine labeled with sulfur-35.

turnover (Doell, 1957) The small amount of activity that enters the follicle later may be a breakdown product from proteins into which the cystine has been synthesized elsewhere in the body. Structural proteins have a low turnover or none at all (Doell, 1957)

After 48 hours the peak has passed out of the skin and the grain densities in that and subsequent samples probably indicate the relative

amounts of sulfur normally present in the different regions (Fig 8). Grain counts (Ryder, 1957b) suggest that the secondary follicles contain the same amount of sulfur as the primary. Grain counts suggest that the two halves of the cortex, associated with the bilateral nature of the wool fiber, contain similar amounts of cystine (Ryder, 1956d). The bilateral nature of wool is associated with the fiber being kera-

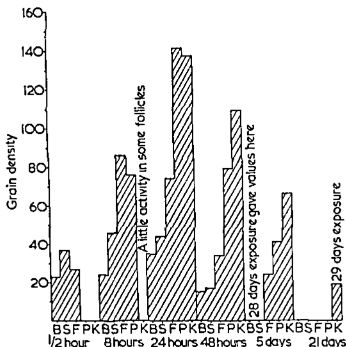


FIG 8 Grain densities in the autoradiographs from different regions of the wool follicle at different times after injection Fourteen days exposure KEY B = Bulb, S = Suprabulbar region, F = Fibrillary region, P = Prekeratinization region, K = Fully keratinized part of fiber (From Ryder, 1957b.)

tinized first at a lower level on one side of the fiber than the other (Auber, 1952) The follicle wall is thinner on the side at which the fiber is keratinized first, and it has been thought that substances are able to reach the fiber more quickly through that side In every sample of skin from the lamb, for a short distance at the upper limit of the autoradiograph, the activity is on one side of the fiber only (Fig 9), suggesting that the active compound has begun to enter that side of the fiber before the other It is unlikely that sulfur enters only from one side, and it enters so quickly that the difference in the thickness of the follicle

wall is unlikely to have any effect. Asymmetry in keratinization therefore seems to be inherent in the follicle.

Sheep can synthesize cystine from sulfate (Hale and Garrigus, 1953), and small amounts of radioactive cystine have been found in the hair of the rat following an injection of labeled sulfate (Dziewiatkowski, 1954) The sheep may be able to synthesize cystine in the follicle (Fraser and Roberts, 1932) Sylvén (1950) found metachromatic staining (polysaccharide ester sulfate) in the papilla and in lower parts of



Fig 9 *Autoradiographic preparation made with Kodak A R 10 film from* thin wax section of skin taken from lamb 48 hours after injection with labeled cystine The preparation was stained with safranin and shows that at the distal limit of the autoradiograph of the fiber, at the skin surface, there is activity in only one (the dye-accessible) half of the cortex Magnification × 400 (From Ryder, 1957b)

the rat follicle. He suggested that this might supply sulfur for keratin synthesis. However, neither Harkness and Bern (1957) nor Ryder (1957b) were able to detect much activity in the follicles of mice injected with labeled sulfate. In sheep, radioactivity was detected after the injection of labeled sulfate, 8 hours afterward activity was present in the bulb and outer sheath of the lower part of the follicle (Ryder, 1957b) (Fig 10) There was some activity up to 5 days after injection

However, activity was found in the fiber after 24 hours and this had reached a level above the skin surface after 3 days. Montagna and Hill (1957) found uptake of labeled sulfate in the rat papilla coincident with metachromasia. The activity found in the bulb and outer sheath is possibly bound in a polysaccharide ester sulfate This is not evidence that polysaccharide ester sulfate gives rise to the active sulfur in the body The results obtained with cystine suggest that sulfur normally enters the follicle in an amino acid, possibly cysteine.



FIG 10 Autoradiographic preparation of thick frozen section of skin from lamb 48 hours after an injection of sulfate labeled with sulfur-35 Most of the radioactivity is in the bulb and outer sheath, with little in the fiber Magnification × 90

C *Copper*

Copper-deficient sheep produce wool that lacks crimp (Fig 11), and according to Marston (1946) the region in the fiber that gives a strong reaction for thiol groups is extended toward the skin surface. He thought that copper probably acts as a catalyst for the oxidation of thiol groups to dithio links There was less cystine (Marston, 1946) and more thiol groups (Burley, 1954) in wool from copper-deficient sheep. Wool

"roots" contained more copper than the shafts (Ellis *et al*, 1950) and copper may be tightly bound in an enzyme (Ryder, 1957b). Rats and mice injected with copper-64 (half life only 12 8 hours) and killed from 1 to 13 hours afterward, do not show localization in the follicles. The copper of ascorbic acid oxidase cannot be exchanged for cupric ion in solution, which indicates a type of bonding close to covalent bonding



Fig 11 Staple of wool from a naturally pigmented Australian crossbred sheep which was copper-deficient when the distal part of the staple was grown (nearest to the bottom of the figure) The wool here lacks crimp and is of a lighter color On dosing with copper sulfate the wool immediately began to grow crimpy and simultaneously acquired more pigment (Staple given by H R. Marston) Reduction × 2/3

(Joselow and Dawson, 1951) This might explain why Ryder (1957b) was unable to detect copper-64 in the follicle

D Glycogen

Glycogen is stored in the outer sheath of the follicle, and is present in the hair (Bolliger and McDonald, 1949) Human hair follicles abound in glycogen (Montagna *et al*, 1951, 1952) Glycogen is found

in the unkeratinized part of the wool fibers of sheep (Ryder, 1956b). It was found lower down on one side than the other.

In discussing the function of the glycogen in the follicle let us first consider that in the outer sheath That this is important for hair growth is shown by the amount becoming reduced during fiber shedding in sheep (Ryder, 1956a), and its absence during the resting stage of the hair growth cycle in other animals (Shipman *et al*, 1955). As long as a hair is growing there is glycogen in the follicle, whatever the nutritional state of the animal (Loewenthal and Montagna, 1955) When the food intake of a mouse is reduced immediately after plucking hairs, hair growth in mice is inhibited, whereas if the animals are plucked 5 to 9 days before restriction of diet, hair growth is only retarded. During the first 6 to 7 days of the cycle the follicle is merely being built up; once the follicle is mature a poor diet could not inhibit hair growth, only retard it, the impetus for hair growth probably being provided by stored glycogen. Sheep kept on a poor diet showed no reduction in the amount of glycogen in the outer sheath (Ryder, 1956a).

Bullough (1952) showed that carbohydrate is essential for mitosis to take place, and the glycogen of the cell becomes diminished during mitosis (Groppe, 1952). Epidermal mitosis in the mouse only occurs when the animal is at rest, when glucose is deposited from the blood stream. There are no such diurnal rhythms in follicle mitosis (Bullough, 1957) In sheep, glycogen is often concentrated in the inner part of the outer sheath (Ryder, 1956b) not in the outer part (Auber, 1952). The lack of glycogen in the outer parts suggests a removal outward, possibly by the surrounding vessel network (Ryder, 1956b)

The presence of glycogen in the fiber suggests that glucose cannot diffuse readily through the follicle wall into the fiber and that the glycogen is stored here to provide energy for keratinization. This is supported by the initial asymmetry of the reaction in wool fibers It is believed that glycogen is needed in epidermal keratinization (Bradfield, 1951). Epidermal cells could store glycogen as they leave the basal layers, and use it later to supply energy for protein synthesis by anaerobic glycolysis. This, however, does not explain the large amount of "unused" glycogen found by Bolliger and McDonald (1948) in fully formed fibers.

In an attempt to throw light on the deposition of glycogen in the follicle six mice were injected with glucose labeled with carbon-14 (Ryder, 1957c). The mice were killed 1 hour, 8 hours, 24 hours, 3 days, and 10 days after injection, the preparations were stained with the modified Hotchkiss method and autoradiographs were made In vibrissal follicles, after one hour, there was a dense autoradiograph of the

bulb with less activity at higher levels (Fig. 12). In the 8-hour and subsequent samples the activity became diminished in the bulb, and there was more activity at higher levels.

In the coat follicles there was activity in the outer sheath of the lower part of the follicle in addition to the bulb. The autoradiographic grain density from the outer sheath seemed to be similar to that from the bulb After 8 hours the situation was similar, but in some follicles there was more activity in the outer sheath than the bulb and this



Fig. 12 Autoradiographic preparation of thick frozen section from mouse killed one hour after an injection of glucose labeled with carbon-14 The bulb of this vibrissal follicle contains much radioactivity Magnification × 105.

coincided with a strong reaction for glycogen There was less activity in the fiber than the surrounding outer sheath After 24 hours the autoradiograph of the outer sheath was clearly denser than that of the bulb and now there seemed to be activity in the inner sheath too Thus, the radioactive glucose seems to be deposited in the outer sheath as glycogen The large amount of activity found in the bulb after one hour is interesting, because this must be glucose or a compound such as glucose-6-phosphate and it is apparently being deposited there to provide energy for mitosis Also, the concentration of activity in the bulb contrasts with the concentration of activity following injection of

labeled cystine, thus supporting the hypothesis that substances enter the follicle at points nearest to the region in which they are required.

E. *The Injection of Other Labeled Compounds*

Leblond (1951) injected newborn rats with carbonate labeled with carbon-14 and found moderate activity in developing hair follicles which were in stages prior to hair formation, 2 and 24 hours after injection, this activity had decreased or disappeared after 3 days

Bern *et al.* (1955) found a similar amount of activity in the bulb and "keratogenous zone" 8 hours after injection of an algal protein-hydrolyzate labeled with carbon-14, and obtained similar results after an injection of labeled carbonate. Harkness and Bern (1957) obtained a slightly greater concentration of activity in the "keratogenous zone" than in the bulb, which they thought suggested that other amino acids in addition to those containing sulfur might be incorporated here too.

Bern *et al.* (1955) and Harkness and Bern (1957) injected mice with phosphate labeled with phosphorus-32 and found activity in the bulb 8 and 16 hours afterward. Activity was evident in the "keratogenous zone" after 24 hours but there was never much activity in the hair shaft. This accords with the incorporation of phosphate into the nucleic acids of dividing cells and the known distribution of ribonucleic and deoxyribonucleic acids (Hardy, 1952).

F *Follicle Competition*

Fraser and Short (1952) considered that differences in follicle and fiber size are due to the competition between follicles for fiber-forming substances from the surrounding tissues The farther apart are any two follicles, the less likely they are to compete Since each follicle is surrounded by a network of blood vessels in the adult (Ryder, 1956c), any competition is likely to be caused by the varying ability of different follicles to draw substances from the blood stream rather than to the distance of one follicle from another Competition in this way is no doubt possible in the fetus, among primary follicles before they get a special blood supply, but the smaller size of the secondary follicles could not be explained by such competition because they are formed later than the primary ones

There is a significant negative regression of the diameter of a fiber in a central position on the sum of the squares of the diameters of surrounding fibers (Fraser and Short, 1952) However, the lack of random arrangement of follicles in the skin must have some bearing on this problem (Ryder, 1957a) since follicles are in groups with the larger, primary follicles on one side and the smaller secondaries on the other

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CHAPTER 15

The Effects of Different Hormonal States on the Growth of Hair in Rats[1,2]

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I. Introduction

Observations on the effects of hormones on hair growth are frequently discordant, incomplete, and limited to spontaneous replacement (Baker, 1951; Cooper, 1930, Houssay, 1953a). The studies presented in this chapter partially clarify the action of gonadal and adrenocortical steroids, epinephrine, thyroid hormone, insulin, and pituitary hormones on hair growth in the rat (Mohn, 1955, 1956, 1957a, b). Spontaneous replacement of hair is compared with growth initiated by plucking hair from resting follicles in gonadectomized, adrenalectomized, hypophysectomized, thyroid hormone-deficient, diabetic, and normal male and female black rats of known ages The effects of various hormone preparations are also observed in these experimental animals

The pilary system of rats readily lends itself to studies of the effects of different hormonal imbalances. In such studies it is imperative, however, that the complexity of the processes of hair growth be understood In the rat and mouse, hair growth is cyclic, that is, hair follicles have alternate periods of activity and quiescence When activity is first initiated, the quiescent follicle grows through the differentiation of a bulb and the other features necessary for the production of a hair When differentiation is completed, it forms a hair. When the hair has grown for a specific period of time, its production suddenly stops, and after a brief period of transition the follicle becomes quiescent. It remains in a resting state for days or months, depending on the age of the animal

The follicles over the entire skin of an animal do not spontaneously become active at the same time In the rat, spontaneous follicular activity begins on the belly and spreads dorsally and posteriorly in a wavelike fashion Of the several waves of growth which occur during the animal's lifetime, the timing of only the first four or five is fairly predictable. Spontaneous waves of growth can be easily followed in pigmented animals if the hair over most of the body is clipped close to the skin Clipping, itself, has no effect on the hair follicles If the

hairs are plucked from resting follicles, however, growth is immediately initiated within the follicles, although it is not known how plucking initiates hair growth Chemical irritation and mechanical injury also stimulate hair follicles to activity (Butcher, 1936, 1940, 1951, Rauch, 1952). Plucking is a useful technique for inducing activity in resting hair follicles and for insuring that the follicles in large areas of skin are at precisely the same stage of activity. Plucking also permits an accurate determination of the time required for complete regrowth of hair under different experimental conditions. Heretofore this technique has been used sparingly in the rat.

It should be stressed that once a follicle becomes active, the cycle of growth is the same regardless of how activity is initiated The early stages of growth are concerned with cell proliferation and the morphogenesis of a growing follicle. The principal activity of a fully differentiated follicle is the production of a hair. After a specific amount of time, *growth ceases and the follicle becomes quiescent again. In order to* study the effects of hormones on hair growth the following features must be considered: (a) the initia [illegible] follicular differentiation, (c) [illegible] extent and pattern of spontaneous replacement, and (f) the quality of the pelage which is produced Whenever investigators have failed to distinguish among these features the results have been discordant.

The present studies were designed with the above mentioned aspects of hair growth in mind. Approximately 540 black rats, ranging in age from one to 6 months, were used (Table I). Litter mates were chosen for each experiment whenever possible, when not possible, animals of the same age were utilized Rats were studied at ages when all the hair follicles on their backs were resting and spontaneous follicular activity had just appeared in the follicles of the belly skin The left side of each animal was then clipped in order to observe the speed and pattern of spontaneous replacement, and the right side was plucked to induce follicular activity experimentally (Fig. 1) After imposing a hormonal imbalance, spontaneous growth and growth initiated by plucking were periodically inspected in each individual Also, the effects of each hormonal imbalance were compared with those of other experimental conditions Body weights were recorded at regular intervals during the experiments, which lasted for a period of 1 to 7 months Care was taken to maintain an adequate nutritional level in adrenalectomized and hypophysectomized rats Hypophysectomized rats received a drinking solution consisting of one part of evaporated milk to two parts of water Adrenalectomized rats received a similar milk solution to which 0 7% NaCl and 0 3% $NaHCO_3$ had been added

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A Hypophysectomy .. ..
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pink skin indicating that follicular development is not far advanced. After 2 or 3 days the skin of the back becomes bluish The color grows progressively darker until the seventh or eighth day, by which time the tips of the hairs are clearly visible above the surface of the epidermis. Differentiation and growth of hair, as detected by the bluish color of the skin, spreads from the dorsum to the head, rump, and belly. Hair proliferation continues until the animal is 3 weeks of age, when the first pelage has completed its growth and all follicles are resting. At 4 weeks the hair follicles of the belly become active again. Follicular activity quickly spreads to the dorsum, and the second coat completes its growth shortly after 7 weeks. The third wave of growth appears on the ventral surface at about the eighth week of age. This wave spreads less rapidly than the preceding one and does not reach the mid-dorsal region for nearly 2 weeks. At 12 or 13 weeks of age the follicles of both the belly and the central portion of the back are resting Those in the sacral region reach this stage at about 14 weeks. The fourth spontaneous wave of growth is present on the abdomen at 13 weeks and is completed at 17 weeks except for a narrow mid-dorsal strip in the lumbar region. Specific observations were not made on the ensuing growth waves; however, spontaneous waves subsequent to the fourth one can be recognized by the presence of pigmented skin, usually in stripes along the length of the body.

Histological examination of skin during the first week after plucking reveals that 4 or 5 days are required for the differentiation of a new hair bulb and the commencement of melanogenesis, whereas the remaining 2 or 3 days are used for hair production and elongation of the follicle. The tips of the hairs emerge about 8 days after plucking, and hairs continue to grow for 18 or 19 days. Growth is completed in most of the follicles of the back about 26 days after the day of plucking. Once an area has been plucked of its hair, the follicles remain out of phase with those in the surrounding skin If a plucked area is left undisturbed after the hair has regrown, a spontaneous growth wave will eventually be established within this area This wave, as well as subsequent waves, spreads in the usual ventral to dorsal fashion, but it is always independent of spontaneous growth waves which occur in the surrounding areas of skin that have not been plucked

B Sex Differences

Although some investigators observe no sex differences in hair growth (Dieke, 1947, 1948), and others report retarded growth in females (Borum, 1954, Emmens, 1942, Fraser and Nay, 1953, 1955), in the present studies the skin and hair of intact rats consistently showed

sex differences (Table II). The juvenile coats of young animals are very similar during the first two waves of growth, but after the third wave the males have a coarser coat of hair than do the females. Confirming the results of Emmens (1942), spontaneous growth waves in female rats tend to lag behind those in intact males; however, the growth cycle of a follicle is the same in both sexes. Endogenous estrogens are probably the main factor in delaying the spontaneous growth

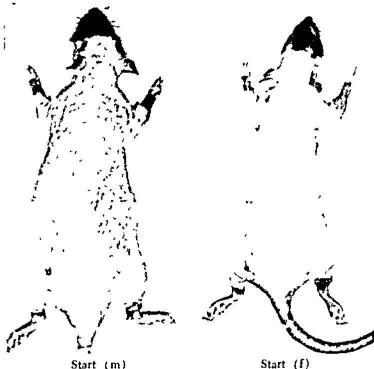


FIG 1 A male (m) and female (f) rat after clipping the right side and plucking the left The darker skin in the male is due to oxidized lipid

waves in females, since similar effects can be obtained by injecting estrogenic substances into castrated animals In addition to these sex differences, the skin of male rats is covered with brownish flakes, presumably oxidized lipid (Dieke, 1947, 1948) These flakes are particularly discernible in animals that have just been clipped and plucked (Fig 1)

C. *The Effects of Pregnancy and Lactation*

Spontaneous growth is inhibited in pregnant and lactating mice (Fraser and Nay, 1953, Nay and Fraser, 1955), and in rats (Table

TABLE II
HAIR GROWTH IN INTACT RATS

Condition of animal	Spontaneous waves			Regrowth after plucking		Hair texture	Remarks
	Frequency	Rate of spread	Growth within waves	Initial response	Remainder of cycle		
Male (normal)	Normal	Normal	Normal	Normal	Normal	Coarse	Thick skin with lipoid scales
Female (normal)	Lag	Slower	Normal	Normal	Normal	Adult but soft	Few scales and skin less thick
Pregnant	Retarded	Retarded	Normal	Retarded	Normal	Normal	
Lactating	Retarded	Retarded	Normal	Retarded	Normal	Normal	
After weaning	Accelerated	Accelerated	Normal	Normal	Normal	Normal	Subsequent waves not accelerated

II). Regrowth after plucking, however, is normal except for a slight delay. The skin of pregnant rats displays little coloration 1 week after plucking, but after 2 weeks the hairs are growing actively. Growth continues at a normal rate and the hairs are fully regrown after 4 weeks. Spontaneous replacement is slightly retarded on the clipped side of pregnant rats, in contrast to the normal replacement in nonpregnant females. After parturition, spontaneous growth is markedly inhibited if the mothers nurse their young (Fig 2). If they do not, however, there is abundant spontaneous growth Induced growth is initially delayed but is otherwise normal in lactating females After weaning the young, the mothers show a marked acceleration of the next spontaneous growth wave (Fig 2).

Consideration of these findings in pregnant and lactating rats suggests that the following hormones could be involved: estrogen and progesterone during pregnancy; luteotropin and progesterone during lactation. Estrogen production is known to increase during gestation but not during lactation (Turner, 1948) Increased estrogen production in pregnant rats may, therefore, account for some of the inhibition of hair growth during pregnancy, since injections of estrogenic substances inhibit hair growth in rats, mice, and dogs (Emmens, 1942; Forbes, 1942, Gardner and DeVita, 1940, Hooker and Pfeiffer, 1943, Houssay, 1953a, b, Mulligan, 1943, 1944, Zondek, 1936) Progesterone also increases during pregnancy, but this hormone does not inhibit hair growth when given to female rats, as will be shown later. Progesterone does, however, delay the replacement of hair in mice (Daneel and Kahlo, 1947) It appears, then, that progesterone is not responsible for the inhibition of spontaneous hair growth in pregnant or nursing rats

Luteotropic hormone (prolactin), in contrast to estrogen, is not abundant during pregnancy, but the level of this hormone increases in the pituitary of pregnant rats just before the young are born and remains high during the period of nursing (Meites and Turner, 1950). Since luteotropin could theoretically account for some of the inhibition of the spontaneous replacement observed during lactation, experiments were conducted to examine the effects of this hormone on hair growth in female rats The results of these experiments are summarized below (also consult Table VII)

When young rats are injected intramuscularly with 10 IU of luteotropin[3] in saline daily, both spontaneous and induced growth appear normal. The waves of growth spread as rapidly as in rats treated with saline alone, and regrowth on the plucked side is normal. Similar ob-

[3] E. R Squibb and Sons



Fig 2 Adult females, clipped and plucked 4 weeks earlier, illustrating the effects of pregnancy and lactation on hair growth Compare the amount of spontaneous replacement during lactation with the replacement after weaning

servations have been reported elsewhere (Emmens, 1942). If, however, adult females that have been nursing for one week are *separated from* their young and placed on luteotropin treatments, spontaneous growth remains slightly inhibited. This inhibition is by no means comparable, however, to the amount of retardation observed in untreated females that continue to nurse their young. The quality of the pelage is not affected, and induced growth is normal.

The reasons for inhibited replacement of hair during pregnancy and lactation are not fully understood Changes in the levels of endogenous estrogen, progesterone, and luteotropin may be responsible. However, differences in the secretory activity of such glands as the thyroid and adrenal should not be overlooked.

III. The Effects of Gonadal Hormones on Hair Growth

A. *Gonadectomy*

There is controversy concerning the effects of gonadectomy in rats and mice Butcher (1934) found that although spontaneous growth is correlated with activity in the rat ovary, ovariectomy produces no change in the appearance of the growth waves. Emmens (1942), on the other hand, stated that spontaneous growth waves in female rats often lag behind those in males and that this sex difference disappears after ovariectomy. Similar observations have been reported in mice (Borum, 1954, Fraser and Nay, 1953, 1955). Dieke (1947, 1948), who found no sex differences in the hair growth of intact rats found a delay in the waves after gonadectomy. Others have observed that whereas castration has no effect on the growth of hair in rats (Baker and Whitaker, 1949, Houssay, 1953a, b, Kozam, 1952), it accelerates the onset of growth in mice (Houssay and Higgins, 1949a). Although this contradiction might be attributed to differences in species and strains (Houssay, 1953a), it seems likely that it is the result of different experimental conditions and criteria, combined with insufficient awareness of the details of hair growth.

Growth of hair on the plucked side is normal in gonadectomized rats of either sex (Table III and Fig. 3) The hair is completely regrown 4 weeks after plucking, however, the hair coat looks and feels intermediate in texture between that of males and that of females The spontaneous hair growth waves seem to spread faster in young ovariectomized rats than in intact females of the same age In orchiectomized animals spontaneous growth waves are similar to those in unoperated males. As in intact animals, spontaneous replacement of hair in castrated animals is slower in older animals

TABLE III
EFFECTS OF GONADAL HORMONES ON HAIR GROWTH

Treatment	Spontaneous waves: Frequency	Spontaneous waves: Rate of spread	Spontaneous waves: Growth within waves	Regrowth after plucking: Initial response	Regrowth after plucking: Remainder of cycle	Hair texture	Remarks
Orchiectomy	Normal	Normal	Normal	Normal	Normal	Intermediate	Few lipoid scales
Ovariectomy	No lag	Like males	Normal	Normal	Normal	Intermediate	Waves resemble those of normal and castrated males
Estrogen	Retarded	Retarded	Retarded	Retarded	Retarded	Fine, sparse	Inhibits growth after gndx Effects more prominent if cortisone given also Inhibition less apparent after adx Effects pronounced after PTU or PTU + adx. + gndx.
Progesterone	Normal	Normal	Normal	Normal	Normal	Normal	No effect in intact rats
Androgen	Normal	Normal	Normal	Normal	Normal	Coarse	Numerous lipoid scales Does not modify effects of cortisone, adx, PTU, or PTU + adx + gndx. Fur remains infantile after hypx.

B. *Estrogen*

It is agreed that estrogenic hormones inhibit hair growth. Injections of estrogens retard spontaneous replacement of hair in rats, mice, and dogs (Emmens, 1942; Forbes, 1942, Gardner and DeVita, 1940, Hooker



FIG 3 Hair growth in gonadectomized (Gndx) rats treated with 50 μg of estradiol benzoate (E B), 200 μg of testosterone propionate (T P.), or 1 mg of cortisone acetate (Cort) for 4 weeks The e is sparse after rogen treatment and coarse after androgen treatment

and Pfeiffer, 1943, Houssay, 1953a, b, Mulligan, 1943, 1944; Zondek, 1936). Even topical applicat ... fective in inhibiting spontaneous hai ... 1942, Whitaker and Baker, 1951a; ... estrogen also produce a reduction in the size of the sebaceous glands and the thickness of the epidermis (Baker and Whitaker, 1949, Ebling, 1953, 1954, Hooker and Pfeiffer, 1943, Ingle and Baker, 1951). This is particularly interesting, since estrogens are said to have a mitogenic effect on the epidermis (Bullough, 1950a, b; Bullough and Van Oordt, 1950). Ebling (1954) has demonstrated, however, that estrogenic substances have independent effects on mitosis and differentiation in both the epidermis and sebaceous glands.

Daily intramuscular injection of 50 µg of estradiol benzoate[4] in oil for 1 month delays and partially inhibits induced hair growth in young castrated rats. The entire growth cycle proceeds more slowly during estrogen treatment, *and the pelage is fine and sparse, particularly in* older animals (Fig 3). There is little or no spontaneous growth in estrogen-treated rats, although in the untreated castrated animals the hairs on the clipped side are fully regrown. If 1 mg of cortisone is given in addition to the estrogen, the inhibition is even more pronounced (Fig 3). When estrogen or estrogen and cortisone treatments are suddenly discontinued, growth commences on both sides of the animal.

In summary, it appears that estrogenic hormones inhibit the initiation of growth in the follicle and prolong the entire growth cycle of the follicle (*Table III*). *The pelage is fine and sparse in estrogen-treated* animals, and the skin is thin and has few lipoid scales. The mechanisms underlying these effects are not fully understood. Presumably, the presence of the adrenal cortex or corticosteroid is necessary for the complete action of estrogens on hair growth (Baker, 1951; Baker and Whitaker, 1949, Ingle and Baker, 1951, Whitaker and Baker, 1951a). The interrelationships of estrogen and adrenocortical hormone are discussed later. However, since small amounts of estrogen applied topically to the skin inhibit hair growth in the treated area (Whitaker, 1956, Williams *et al.*, 1946), it seems likely that there are some direct effects on the skin and hair follicles Capillary fragility is decreased during estrogen treatment (Kozam, 1952), and perhaps less nutritive material reaches the hair follicle than under normal circumstances. Similarly, since vitamin B_{12} counteracts the inhibition of body weight by diethylstilbestrol (*Meites and Shay, 1951) and that vitamins are often* components of enzyme systems, estrogens might exert their effects on hair

[4] Ciba Pharmaceutical Products

growth by inhibiting certain enzyme systems within the skin or hair follicles.

C. Progesterone

Daily intramuscular injections of 50 or 200 μg of progesterone[5] in oil have little if any effect on spontaneous and induced hair growth in 8-week-old female rats (Table III). Spontaneous waves appear to spread as rapidly in rats injected with progesterone as in rats receiving oil alone. Also, the cycle of induced growth is normal Similar results have been obtained by Emmens (1942) and Kozam (1952). Moderately large doses have no effect on hair growth in dogs (Trentin *et al*, 1952) but inhibit the replacement of hair in mice (Daneel and Kahlo, 1947).

D. Androgen

The role of androgens in hair growth is not clear Androgens are said to "protect" hair growth against estrogenic inhibition in the rat (Hooker and Pfeiffer, 1943) but not in the dog (Gardner and DeVita, 1940). Androgens are the incitant factors in human male alopecia (Hamilton, 1942) Testosterone usually has no effect on the amount of spontaneous replacement in rats (Emmens, 1942, Kozam, 1952), but moderately large doses inhibit growth (Forbes, 1942, Houssay, 1953a) Even small amounts of androgen inhibit replacement in mice (Houssay, 1953a). These inhibitory effects, however, may be the result of a generalized toxic reaction rather than a physiological response

In the present studies, hair growth is normal on the plucked side of gonadectomized animals treated intramuscularly with 200 μg of testosterone propionate[6] in oil (Fig. 3). Four weeks after plucking, a heavy coat of predominantly coarse hair is fully grown, and the thick skin is covered with brownish flakes. There is little difference between the amount of spontaneous growth in the androgen-treated castrated animals and that in the untreated castrates of the same age If, however, 1 mg of cortisone is given in addition to the testosterone, spontaneous growth is markedly reduced (Fig 3) Growth on the plucked side remains normal but initially delayed, and the pelage is coarse and like that of normal males Stopping of the combined androgen-cortisone treatment is followed by spontaneous growth, which quickly spreads over the clipped side Testosterone also has no effect in adrenalectomized, hypophysectomized, or thyroid hormone-deficient animals, as will be described later

In the rat the effects of androgen are limited to the production of a coarse pelage and a thick skin covered with flakes of oxidized lipid

[5] Ciba Pharmaceutical Products
[6] Ciba P[illegible]aceutical Products

(Table III). Androgen has no effect on capillary fragility in the skin of rats (Kozam, 1952), but it increases cutaneous vascularity in human beings (Edwards *et al*, 1941, Hamilton, 1939) Although testosterone exerts a mitogenic effect on the epidermis of mice, rats, and rabbits (Bullough, 1952b; Bullough and Van Oordt, 1950, Eartly *et al*, 1951, Montagna *et al.* 1949) it has no apparent effect on the hair follicles.

IV. The Effects of Adrenal Hormones on Hair Growth

A Adrenalectomy

Adrenalectomy induces quiescent follicles to become active in rats and mice, and it is said to "accelerate" hair growth (Baker and Whitaker, 1949, Butcher, 1937, Houssay, 1953a; Ingle and Baker, 1951; Dieke, 1948; Kozam, 1952). After growth is completed in the follicles, the ensuing quiescent period is short and the subsequent waves of replacement are also accelerated (Baker, 1951; Dieke, 1948). Adrenalectomy accelerates hair growth even in animals with follicles markedly inhibited by reduced food intake (Butcher and Richards, 1939), pantothenic acid deficiency (Ralli and Graef, 1943, 1945), or copper deficiency (Hundley and Ing, 1951). It is not clear from previous studies whether the entire growth cycle has been affected by adrenalectomy or merely the initiation of growth The observations reported here provide an answer to this question (Table IV).

Hair growth induced by plucking on the day of adrenalectomy is similar in adrenalectomized and sham-operated animals (Figs. 4 and 5). Growth is not detected earlier than usual, and the hair follicles do not complete their growth cycle until the usual 4 weeks after plucking If the club hairs are again plucked 1 month after the operation, the growth which is initiated proceeds at a normal rate and is completed within 4 weeks. Spontaneous growth, however, commences immediately after adrenalectomy, regardless of the age of the animal Instead of progressing in a ventrodorsal wave as in intact animals, follicular activity commences almost simultaneously on the entire clipped side. Two weeks after the operation the hair on the unplucked side has grown nearly as well as that on the plucked side, whereas in sham-operated animals spontaneous growth has spread in a wavelike fashion (Fig. 4). After 4 weeks, all growth is complete on the clipped side of the adrenalectomized rats, but the hair is still growing in control animals (Fig 5) If the left side is again clipped 4 weeks after adrenalectomy, the next spontaneous replacement proceeds in a wavelike fashion, but, it spreads more rapidly than in sham-operated animals

It is important to emphasize that after adrenalectomy only the initiation of growth is accelerated, and the time required to produce

TABLE IV

EFFECTS OF ADRENAL HORMONES ON HAIR GROWTH

Treatment	Spontaneous waves: Frequency	Spontaneous waves: Rate of spread	Spontaneous waves: Growth within waves	Regrowth after plucking: Initial response	Regrowth after plucking: Remainder of cycle	Remarks[a]
Adrenalectomy	Accelerated	Accelerated	Normal	Normal	Normal	Subsequent waves also accelerated Regrowth after plucking always normal
Cortisone						
1 mg/d in intact rats, gndx rats, adx rats	Retarded	Retarded	Normal	Retarded	Normal	No cumulative effects No "refractoriness"
1 mg/d in PTU rats, PTU + adx + gndx, hypx rats	Prevented	Prevented	Normal	Prevented	Prevented[b]	Prevents all growth except in existent growing follicles
10 mg/d in intact rats	Prevented	Prevented	Normal	Prevented	Prevented[b]	Prevents all growth except in existent growing follicles
Deoxycorticosterone	Normal	Normal	Normal	Normal	Normal	Does not affect growth in intact or adx rats
Epinephrine	Retarded	Retarded	Normal	Retarded	Normal	Local inhibition at site of injection and white hairs Only local effects found after adx. Action of epinephrine not influenced by presence or absence of thyroid hormone Effects more pronounced if cortisone also given to adx. or PTU rats

[a] Texture of hair normal for all treatments

[b] See Table VIII.

a hair remains unchanged. This acceleration of follicular activity is a direct result of the lack of adrenocortical hormones (Butcher, 1937); changes in the output of thyroid or pituitary hormones after adrenalectomy are not significantly involved (Butcher, 1941; Zeckwer, 1953a). However, Parnell (1953) has observed a greater acceleration after adrenalectomy in male rats than in females and more acceleration in

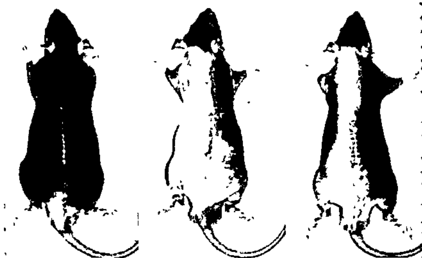




FIG. 4 Male rats adrenalectomized (Adx) for 2 weeks Hair growth is less advanced in the estrogen-treated rat than in its untreated littermate Androgen and ACTH show no effect. Cortisone prevents the acceleration of spontaneous growth induced by adrenalectomy

normal animals than in castrates. If one of a pair of parabiotic rats is adrenalectomized, there is immediate hair growth in its skin but no change in hair growth in the skin of its intact partner. If, however,

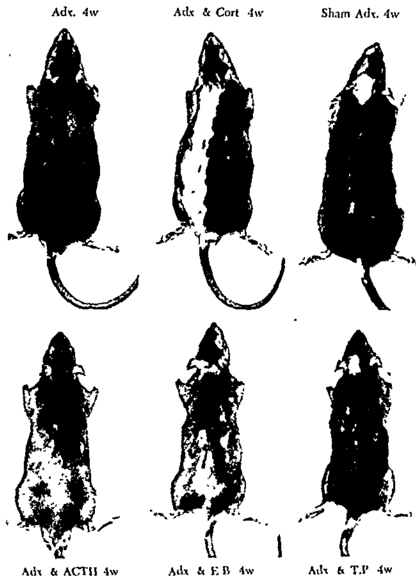


FIG. 5 Adrenalectomized (Adx) rats injected daily with 1 mg of cortisone acetate, 250 μg of ACTH, 50 μg of estradiol benzoate (E B), or 200 μg of testosterone propionate (T P) for 4 weeks. Cortisone has inhibited spontaneous replacement only, whereas all the hair is still growing in the estrogen-treated animal. The pelage is coarse in the rat receiving androgen

salt is later withdrawn from the water, the intact rat shows signs of adrenal hypertrophy and its hair growth is retarded. At the same time, initiation of growth is no longer accelerated in the adrenalectomized partner (Zeckwer, 1953b). The adrenal cortex must normally exert constant restraint on the initiation and the amount of spontaneous follicular activity but has no effect on hair growth once initiated.

B. *Cortisone*

Treatments with cortisone and similar corticosteroids inhibit hair growth in rats and mice (Baker, 1951; Baker and Whitaker, 1948; Houssay, 1953a; Meites, 1951; Ralli and Graef, 1945, Whitaker and Baker, 1948, 1951a, b). However, only the initiation of growth and early stages of follicular development are affected by cortisone, once started, both spontaneous and induced hair growth proceed unhampered (Table IV). Daily intramuscular injection of 1 mg of cortisone acetate[7] in a saline suspension has little effect on the growth induced by plucking of intact, gonadectomized, or adrenalectomized rats (Figs. 3–6). After an initial delay of 1 or 2 days the pelage develops at a normal rate and is fully grown 4 weeks after plucking. If the hairs are plucked again, they regrow normally. Spontaneous growth, however, is markedly inhibited by cortisone treatments Only scattered areas become active, but once growth has started it proceeds at a normal rate Females usually have less spontaneous growth than males Cortisone does not affect the quality of the hair produced or the amount of melanin in the hair

The inhibitory effect of cortisone on the initiation of hair growth seems to be a direct effect on the hair follicles and skin, since only those follicles in the treated area are affected by the topical application of cortical hormones (Baker *et al*, 1948, Whitaker and Baker, 1948, 1951a, b). An appreciable amount of tritium-labeled cortisone reaches the skin of rats from 3 to 8 minutes after injection, but the level quickly falls and most of the cortisone eventually reaches the liver where it is metabolized (Bradlow *et al*, 1954).

Reports that hair follicles become "refractory" to cortisone (Baker, 1951, Castor and Baker, 1950) have not been substantiated by the present investigation (Table IV) Nor is there any cumulative effect of cortisone on hair growth Intact rats were injected intramuscularly with 1 mg of cortisone daily for 7 months, yet follicles plucked and allowed to regrow 7 times produced good hair each time (Fig 6) Spontaneous growth waves were retarded throughout the entire period of cortisone treatment Hair is very gradually replaced spontaneously during prolonged treatment with cortisone, but once activity has com-

[7] Merck and Company



FIG. 6 The effect of stopping cortisone injections after prolonged treatment in intact rats Each rat was plucked on one side and clipped on the other after 6 months of treatment Observe the abundant spontaneous growth in rats receiving only saline for 4 weeks and the absence of spontaneous replacement in rats continued on cortisone Induced growth was essentially normal in each animal

menced in a particular area of skin it goes through its cycle at a normal rate. When, after several months of treatment, cortisone injections are suddenly discontinued and the animals are clipped and plucked, spontaneous growth begins at once on the belly and proceeds to the dorsum in a wavelike fashion (Fig. 6). Hair growth induced by plucking remains normal The cortisone-treated, control rats usually show no spontaneous growth during this period, and normal rats of the same age never have as much spontaneous replacement.

Larger amounts of cortisone have a more pronounced effect on hair growth of 8-week-old rats (Table IV). Animals injected with 5 or 10 mg daily for 4 weeks show no spontaneous replacement, and growth after plucking is confined to a few isolated hairs or groups of hairs (Fig 7). The skin of these animals is very thin. In animals treated with 2 mg of cortisone, delay of growth induced by plucking is noticeable even after 2 weeks of treatment and spontaneous growth is practically stopped. After 4 weeks, the hair on the plucked side is almost fully regrown, but growth is restricted to the belly on the clipped side. As described earlier, after 1 mg of cortisone, growth initiated by plucking is normal except for a slight initial delay. Spontaneous growth is inhibited but less so than in older animals receiving 1 mg of cortisone In control rats treated with the saline vehicle alone for 4 weeks, growth is completely normal. If after 4 weeks the hormone injections are stopped and the backs are clipped, both sides of the cortisone-treated animals show abundant activity after 1 week, and after 4 weeks the entire pelage has nearly regrown on both sides Progressively greater inhibition of spontaneous replacement also occurs when larger amounts of cortisone are applied topically (Whitaker and Baker, 1951b).

Although cortisone reduces mitotic activity in the epidermis (Baker and Whitaker, 1948, Bullough, 1952a), it does not do so in the hair follicle Once sufficiently started, growth in the follicle is not affected by cortisone treatments Capillary permeability and fragility increase after adrenalectomy (Britton, 1930, Kozam, 1952) Conversely, then, cortisone should decrease vascular permeability. Any change in the permeability of capillaries supplying the follicles could either decrease the availability of nutritional elements essential for the initiation of growth or fail to remove some "inhibitory agent" (Chase, 1955) from the follicle Cortisone could force a reduction in some building block required for the initiation of hair growth through its effects on the general metabolism of carbohydrate, protein, and fat Lastly, inhibition of hair growth could be the result of enzyme inhibition in the skin or hair follicles Vitamins, minerals, and sulfhydryl groups are known components of enzyme systems, and changes in either the amounts of these









Fig 7 Compariso[illegible] of hair growth in [illegible] treated with 1, 2, 5, or 10 mg of cortisone acetate [illegible] 4 we[illegible] [illegible] the progressive inhibition of spontaneous growt[illegible] [illegible] ent[illegible]d with 5 or 10 mg of cortisone

components or in the amount of adrenal steroids will affect hair growth (Anderson *et al.*, 1951, Hundley and Ing, 1951; Meites, 1951; Ralli and Graef, 1943, 1945). For example, cortisone inhibits the synthesis of chondroitin sulfate by the skin (Layton, 1951, Schiller and Dorfman, 1957). This acid mucopolysaccharide is normally present in the dermal papilla and the internal sheath of growing hair follicles but absent in resting follicles (Montagna *et al*, 1952).

C. *Deoxycorticosterone*

In contrast to cortisone and other corticosteroids, deoxycorticosteroids have little or no effect on skin and hair growth in rats and gonadectomized mice (Emmens, 1942, Houssay, 1953a; Houssay and Higgins, 1949b) (Table IV). Daily or weekly intramuscular injections of 1 mg of deoxycorticosterone acetate[8] in oil per week or per day for 1 month have no effect on growth initiated by plucking or on spontaneous replacement of hair in intact rats (Fig 8). Similarly, intramuscular treatment with a saline suspension of deoxycorticosterone trimethylacetate[8] produces no effect on hair growth, whether given as a single initial injection of 5 mg or as weekly injections of 5 mg (Fig. 8) The quality of the fur is also normal after either hormonal preparation Furthermore, the hair follicles of adrenalectomized rats do not appear to be affected by deoxycorticosteroids When adrenalectomized rats are treated with 5 mg of deoxycorticosterone on the day of the operation, induced hair growth is normal and spontaneous replacement remains accelerated, as in untreated adrenalectomized rats. Cortisone-treated animals, on the other hand, do not have an accelerated replacement of hair. Some investigators have reported an inhibition of hair growth during deoxycorticosteroid treatment (Baker, 1951, Ralli and Graef, 1945, Whitaker, 1949). These investigators used different experimental conditions and different criteria for hair growth. Although electrolyte metabolism is the primary target of the deoxycorticosteroids, they also have some effect on carbohydrate metabolism, and this could exert an inhibitory tendency. Regardless of these considerations, even relatively large doses of deoxycorticosteroids have decidedly less effect on hair growth than do cortisonelike compounds (Whitaker and Baker, 1951b) Interestingly, deoxycorticosterone has little or no effect on the mitotic activity of the epidermis (Bullough, 1952a)

D *Epinephrine*

Numerous effects of epinephrine would suggest possible influences on the hair follicle This hormone produces a general disturbance in

[8] Ciba Pharmaceutical Products



FIG 8 Intact rats treated with 1 mg of deoxycorticosterone acetate (DOCA) per day or 5 mg of deoxycorticosterone trimethylacetate (DOCA³) each week After 4 weeks neither treatment shows any effect on hair growth

carbohydrate metabolism, marked hyperglycemia, and decreased glucose uptake in various tissues (Sutherland, 1951), and in low concentrations it decreases capillary permeability (Gellhorn, 1933), increases capillary resistance (Kozam, 1952, Lavollay, 1944), and is a cutaneous vasoconstrictor. Epinephrine also stimulates other endocrine glands which, in turn, affect skin and hair growth. From experiments with implanted cortical tissue in adrenalectomized rats, Butcher (1937) concluded that a lack of medullary tissue has no effect on hair growth, but others (Stein and Wertheimer, 1941) have observed an increased hair loss in medullectomized rats, and a decreased loss of hair in epinephrine-treated animals.

Two preparations of epinephrine were used in these studies. Some animals were given an aqueous solution of epinephrine[9] subcutaneously, others received intramuscular injections of this hormone in an oil suspension[10] The dosage for either preparation was 0.1 mg twice daily, administered either to the right or to the left hind leg Regardless of other hormonal treatments, all rats receiving epinephrine gain weight less rapidly than comparable untreated animals and usually show signs of hyperglycemia and glycosuria. Oil frequently accumulates in the hind legs of some rats treated with epinephrine in oil, and sterile abscesses occasionally develop. Abscesses rarely form during treatment with the aqueous preparation, but a small necrotic area of skin appears if the injections are administered frequently at the same site The results were similar for both preparations of epinephrine The results of the different experiments are summarized in Table IV

When intact rats of either sex are treated with 0 1 mg of epinephrine in oil twice daily, the initial response of the plucked follicles is markedly retarded (Fig 9). After the initial delay, however, growth of these follicles is normal, and the hairs are fully grown after 4 weeks. If the resting hairs are again plucked, regrowth is normal except for another initial delay. Regrowth after plucking is normal in oil-treated control animals Epinephrine markedly inhibits spontaneous replacement on the clipped side (Fig. 9). Follicular activity spreads much more slowly than in control animals, and the site of injection appears to be "by-passed", that is, *no growth is seen near the hind leg which received the hormone,* although spontaneous activity slowly spreads dorsally and posteriorly When treatment is continued for 4 more weeks, spontaneous growth eventually reaches the skin of the leg, but the hairs are white Similar nonpigmented hairs are found at the site of epinephrine injection regardless of other hormonal imbalances This effect on pigmentation is

[9] Lederle Laboratories

[10] Park Davis and Company

not merely due to the mechanical injury sustained by frequent injections, since the animals receiving the carrier solution rarely grow white hairs in this area. Epinephrine also produces local effects when injected into the leg on the plucked side.

Epinephrine is poorly tolerated by adrenalectomized rats, and many of the animals die within 2 weeks Only two rats in these experiments

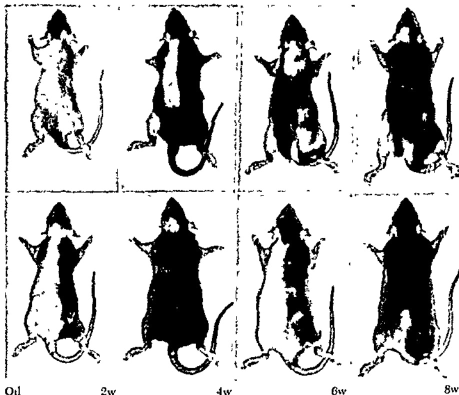


FIG 9 Two female littermates illustrating the effects of epinephrine during 8 weeks of treatment Both rats clipped and plucked again after 4 weeks Observe the inhibition of spontaneous growth in the epinephrine-treated animal and the delay in response to plucking Local effects are seen in both hind legs of this animal, white hairs are clearly visible after 8 weeks of treatment.

survived more than 4 weeks of injections. The animals usually display hyperglycemia and glycosuria, and they gain less weight than untreated adrenalectomized rats Despite poor health, however, regrowth of hair after plucking is normal in adrenalectomized rats treated with epinephrine (Table IV) There is no initial delay in response to plucking,

and the hairs are fully grown 4 weeks later (Fig 10). In addition, injections of epinephrine do not affect the accelerated replacement of hair on the unplucked side. Growth commences over this entire side immediately after adrenalectomy, and the hairs are fully regrown after 4 weeks of epinephrine treatment, as in untreated animals. Growth is inhibited locally, however, in the skin of the two hind legs where the hormone was administered When 1 mg of cortisone acetate is administered intramuscularly three times each week in addition to the epinephrine, there is a pronounced delay in the response to plucking, but once the follicles have developed sufficiently, the rate of growth is normal (Fig. 10). Spontaneous activity on the clipped side spreads in a wavelike fashion, but more slowly than that of adrenalectomized rats receiving cortisone alone. The local inhibitory effects of epinephrine are especially prominent in these animals.

In summary, continuous treatment with epinephrine inhibits spontaneous hair growth in intact rats and delays the response to plucking Once growth has started in a follicle, it proceeds normally. Unlike the epidermis (Bullough, 1952a, 1955, Chaudhry *et al.*, 1956), the epinephrine has no effect on the mitotic activity of hair follicles, and the rate of growth is normal once the follicles have become active. Prolonged treatment with epinephrine also produces a local inhibition of hair growth, whether spontaneous or induced, growth waves tend to by-pass the area of injection, and induced growth is locally retarded When hairs eventually grow near the sites of injection, they have no pigment. The role of epinephrine in melanin production is not clearly understood (Lerner and Fitzpatrick, 1950, Lerner *et al*, 1954).

The inhibitory effects of medullary hormone on hair growth are probably not due to hyperglycemia (Bullough, 1952a, 1955), but may be caused in part by a change in the vascular system The oxidation product of epinephrine, adrenochrome, may also be involved in these results (Bullough, 1952a, 1955). Data to be presented later in this chapter indicate that the effects of epinephrine on hair growth are neither mediated nor potentiated by thyroid hormone. They are, however, partially linked to adrenocortical activity, epinephrine inhibits hair growth more in cortisone-treated adrenalectomized rats than in adrenalectomized rats not receiving cortisone

E Relationship of Adrenocortical Hormones to Gonadal Hormones

1. Cortisone and Estrogen

Injections of estrogen stimulate the adrenal cortex directly and indirectly via the hypophysis and adrenocorticotropin It is entirely possible that some of the inhibition observed in estrogen-treated animals

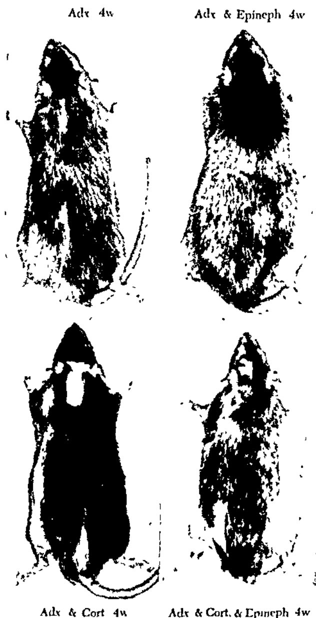


FIG 10 Adrenalectomized (Adx) rats treated with 0 1 mg of epinephrine twice each day, 1 mg of cortisone acetate every other day, or a combination of the two treatments. Treatment with epinephrine alone produced local effects only, cortisone restored the wavelike replacement of hair, injection of both hormones had more effect than injection of either alone

may be due to an increased level of endogenous cortical hormone. Such a mechanism cannot be the complete explanation, however, since topical applications of estrogenic compounds also produce local effects (Whitaker, 1956, Williams *et al*, 1946). Furthermore, estrogen not only inhibits spontaneous growth and delays the response to plucking in intact rats, but, in contrast to cortisone, it prolongs the entire cycle of growth and produces a fine, sparse pelage.

It has also been suggested that adrenal hormones potentiate or facilitate the action of estrogen on hair growth and that estrogens have little effect in the absence of cortical hormones (Baker, 1951, Baker and Whitaker, 1949, Ingle and Baker, 1951, Whitaker and Baker, 1951a). In the present investigation hair growth is particularly inhibited when gonadectomized rats are injected with cortisone in addition to estrogen (Fig 3). However, estrogen also tends to inhibit hair growth even in adrenalectomized rats (Table III). Daily intramuscular injection with 50 μg of estradiol benzoate in oil is usually fatal to adrenalectomized rats, but a sufficient number survive to permit a determination of the effects on hair growth. The skin on the plucked side of these animals shows little coloration after 1 week, whereas it is deeply colored in rats not receiving estrogen. After 2 weeks the estrogen-treated animals consistently have less induced growth than do untreated adrenalectomized rats or sham-operated animals (Fig. 4). After 4 weeks of treatment, the follicles have not completed their growth, and the hair is soft and sparse (Fig 5). Spontaneous growth is also affected in these animals. During the first 2 weeks of estrogen treatment spontaneous activity tends to spread in a wavelike manner, whereas in adrenalectomized rats not receiving estrogen it commences simultaneously over the entire side After 4 weeks of treatment, the clipped side is covered with soft hair, much of which is still growing.

Estrogen produces several effects on the skin independent of adrenocortical hormones. When animals are adrenalectomized, all the resting hair follicles become active and the effects of estrogen are less apparent. Parnell (1953) has reported that adrenalectomy accelerates spontaneous growth more in male rats than in female rats, and Ingle and Baker (1951) have observed some inhibition of hair growth in adrenalectomized rats treated with estrogen. Similarly, Bullough (1952b) has described mitogenic effects with estrogen on the epidermis of adrenalectomized mice The present investigation and the work of Whitaker (1956) show that estrogen affects the entire cycle of growth in the follicle and has an action independent of that of the adrenal cortex. Whitaker (1956) observed a local inhibition of hair growth in guinea pigs receiving topical applications of α-estradiol Since most of the

follicles are growing at any given time in the guinea pig, estradiol must inhibit growing follicles as well as those just beginning to grow. Cortisone, on the other hand, has no effect on the hair follicles of the guinea pig.

2 *Cortisone and Androgen*

The effects of androgen are not mediated by adrenocortical hormone (Table III). Daily intramuscular treatment with 200 μg of testosterone propionate in oil does not affect the growth of follicles plucked on the day of adrenalectomy Follicular activity is detected at the same time as that of adrenalectomized rats not receiving androgen, and hair growth is complete after 4 weeks (Figs 4 and 5). Animals treated with testosterone, however, have coarse hair typical of males. Male hormone has no effect on spontaneous replacement, the follicles become active immediately after adrenalectomy, as do those of untreated rats, and the *pelage is completely grown in 4 weeks.*

V THE EFFECTS OF THYROID HORMONE ON HAIR GROWTH

A. *Thyroidectomy and Goitrogenic Compounds*

Thyroidectomy is reported to inhibit hair growth in rats (Baker, 1951, Butcher, 1941, Chang, 1926, Chang and Feng, 1929, Dieke, 1948, Emmens, 1942, Zeckwer, 1953a) and to alter the pattern of spontaneous replacement (Dieke, 1948) Freud (1934) found, however, that hair growth is not inhibited when thyroidectomized rats are plucked Certain goitrogenic substances also inhibit hair growth in rats (Dieke, 1947), others produce a graying of the hair but little inhibition of growth (Dieke, 1947, Richter and Clisby, 1941). The present studies extend these observations and clarify several details of hair growth in animals lacking thyroid hormone Animals were made deficient in thyroid hormone with propylthiouracil (PTU) Each rat was given a single subcutaneous injection of 10 mg of PTU per 100 grams of body weight, from a stock solution of 2% PTU and 5% gum arabic in aqueous solution. After the injection of PTU the animals were maintained on a drinking solution of 0 02% PTU in water

Except for an initial delay, induced hair growth is normal in rats treated with PTU, and the quality of the pelage is similar to that of normal animals (Table V) One week after plucking, the skin appears uncolored, but after 2 weeks the hairs have emerged a little above the surface of the epidermis (Fig 11) Growth is normal thereafter, and the hairs are fully grown 4 weeks after plucking (Fig 12) If the hairs are again plucked, they regrow normally within 4 weeks except for another initial delay Spontaneous growth, on the other hand, is re-

PTU 2w | PTU & Thy 2w | Norm 2w









PTU & Cort 2w | PTU & E B 2w | PTU & T P 2w

Fig 11 Rats made thyroid hormone-deficient by treating for 2 weeks with propylthiouracil (PTU) Growth is delayed on the plucked side of the animal receiving PTU only but is normal in the rat receiving thyroxine (Thy) Androgen has neither stimulated nor inhibited hair growth Growth has been greatly delayed in the estrogen-treated rat No regrowth is observed in the rat treated with 1 mg of cortisone daily

tarded for the entire period of study. Follicular activity spreads very slowly, but once a follicle becomes active the cycle of growth is normal.

A deficiency of thyroid hormone, therefore, inhibits only the initiation and the early stages of hair growth Once growth has started, whether spontaneous or induced by plucking, the follicles are not affected. No reversal of pattern has been observed in PTU-treated animals, except for a few rats maintained on PTU for long periods of time. Any change in pattern is probably due to influences not directly related to thyroid hormone In contrast to other goitrogenic substances (Dieke, 1947, Richter and Clisby, 1941), PTU does not affect hair pigmentation in the rat, and produces no change in the texture of the fur. Although rats are reported to have an infantile pelage if thyroidectomized when young (Scow *et al*, 1949), and congenitally hypothyroid children frequently retain their lanugo hair (Ravera *et al.*, 1956), these findings probably can be interpreted as retention of infantile hair owing to inhibition of normal hair replacement.

B Adrenalectomy and Gonadectomy in Thyroidectomized Animals

Adrenalectomy accelerates hair replacement in thyroidectomized animals (Butcher, 1941, Zeckwer, 1953a). One report states that adrenalectomy accelerates growth less in thyroidectomized rats than in intact animals (Zeckwer, 1953a), another reports the same amount of acceleration in thyroidectomized and intact rats (Butcher, 1941) Actually, adrenalectomized-gonadectomized rats receiving PTU show less acceleration of hair growth than do animals with intact thyroids (Table V)

Several litters of young rats were placed on continuous PTU treatment as previously described Since preliminary attempts to remove both adrenals on the same day were unsuccessful, adrenalectomy was performed in two stages. One adrenal and one ovary (or both testes) were removed after several days of PTU treatment The second adrenal and ovary were removed approximately 3 weeks later, at which time the animals were plucked on one side and clipped on the other side The results described below are based on reasonably healthy animals that survived longer than 1 week (Tables I and V).

Except for an initial delay, regrowth on the plucked side is normal in rats deprived of adrenal, gonadal, and thyroid hormones One week after the removal of the second adrenal, the skin of the plucked side is barely colored, whereas it is darkly colored in animals not receiving PTU After 2 weeks the hairs have barely emerged; after 4 weeks they are fully grown The texture of the fur is intermediate between that of males and females Spontaneous replacement of hair is delayed in

comparison with replacement in rats lacking only their adrenal glands, but after the initial delay the entire unplucked side responds as a unit and is almost completely regrown 4 weeks after the removal of the second adrenal. Similar observations were made on rats which were adrenalectomized and placed on PTU several weeks later. Induced growth was normal but initially delayed after PTU was given, and there was less acceleration of spontaneous replacement (Fig. 13).

C. *Thyroxine*

Although it is believed that thyroxine hastens the growth of hair in rats (Butcher, 1937, 1940, Kozam, 1952), Emmens (1942) reports that it does not affect hair growth in rats, and Chang (1926) claims inhibition of growth in hyperthyroidism. Daily intramuscular treatment with 20 µg of thyroxine[11] in saline accelerates spontaneous growth waves in 12-week-old intact rats but has no effect on growth initiated by plucking. Spontaneous activity spreads more rapidly than in untreated animals, and subsequent waves appear sooner when treatment is continued for 8 weeks, the cycle of growth in each follicle remains normal regardless of how growth is initiated. When 10 µg of thyroxine are administered daily to PTU-treated rats, induced growth and spontaneous replacement are normal (Figs. 11 and 12). In adrenalectomized-gonadectomized rats receiving PTU, daily treatment with 20 µg of thyroxine eliminates the delay found in animals not treated with thyroxine. The skin is well pigmented 1 week after plucking, the hairs are fully grown within 4 weeks, and the pelage resembles that of gonadectomized rats Spontaneous growth in these thyroxin-treated animals is accelerated, and is similar to that found in rats lacking only their adrenals Activity commences immediately after the removal of the second adrenal, and nearly all the hairs are fully grown after 4 weeks

Thyroxine, then, stimulates the spontaneous replacement of hair in intact and thyroid hormone-deficient animals (Table V) The cycle of growth remains normal regardless of how activity is initiated in the follicle The mechanism of thyroid action on hair growth is not known, but thyroxine treatments are known to increase capillary fragility in the skin (Kozam, 1952), this may enhance the utilization of essential nutrients. Thyroxine produces a local stimulation of the feather papilla of hens (Juhn and Harris, 1955) and may have a similar action on the hair follicles of rats It also increases the amount of acid and alkaline phosphatase in the skin and feathers of pigeons (Kobayashi *et al*, 1955). Thyroxine decreases oxidative phosphorylation in preparations of liver mitochondria (Beyer, 1956, Martius and Hess, 1951) but it is said to

[11] E R Squibb and Sons

enhance succinoxidase activity (Lipner and Barker, 1953). Perhaps thyroxine stimulates the skin and hair follicles directly and acts at the mitochondrial level of organization on oxidative enzyme systems.

D. *Relationship of Thyroid Hormone to Gonadal Hormones*

1. *Thyroxine and Androgen*

Thyroxine and testosterone are said to be antagonists in maintaining the epidermis of rats (Eartly and Grad, 1951; Eartly *et al*, 1951; Eartly and Leblond, 1951). Thyroxine produces a thin epidermis with decreased mitotic activity, whereas testosterone has the opposite effect Combined treatment with thyroxine and testosterone produces effects intermediate between those of the two hormones. Antagonism has not been found in hair growth (Table III). Daily intramuscular treatment with 200 μg of testosterone propionate has no effect on growth initiated by plucking PTU-treated rats (Figs 11 and 12), the hairs emerge at the same time as those of rats not receiving androgen, and the coat is fully grown after 4 weeks of treatment The pelage feels thick, it is glossy and coarse, and the skin is covered with flakes of oxidized lipid Spontaneous replacement remains inhibited during androgen treatment, as in PTU-treated animals receiving no testosterone (Figs. 11 and 12). Male hormone is also ineffective on hair growth of adrenalectomized-gonadectomized animals treated with PTU, except for producing a coarse pelage, spontaneous growth is accelerated to the same degree as that of rats not receiving androgen, and induced growth is normal The skin, however, is covered with flakes of lipid.

2. *Thyroxine and Estrogen*

Estrogen does not require the presence of thyroid hormone for its effects on hair growth Intramuscular injection with 50 μg of estradiol benzoate each day noticeably retards growth on the plucked side of PTU-treated animals (Table III) After 1 week, there is no coloration of the skin, and after 2 weeks the tips of the hairs are less visible than those of rats on PTU alone (Fig. 11). Hair continues to grow more slowly in the estrogen-treated animals, and it is still growing after 4 weeks of treatment (Fig 12), the skin is thin and the fur is soft and sparse As in untreated animals, there is no spontaneous hair growth during the 4 weeks of estrogen treatment These effects are not dependent on the presence of adrenocorticosteroids Estrogen delays the response to plucking and prolongs the entire cycle of growth even in adrenalectomized-gonadectomized rats receiving PTU The follicles have not completed their growth 4 weeks after plucking, the pelage is fine and sparse, and, the skin is thin and has few flakes of lipid. Animals

receiving no female hormone have abundant spontaneous replacement, whereas estrogen-treated rats show little growth Only small patches of hair are found on the clipped side after 4 weeks of injection.

E Relationship of Thyroid Hormone to Adrenal Hormones

1 Thyroxine and Cortisone

Thyroxine and cortisone have opposite effects on hair growth Daily intramuscular injection of 1 mg of cortisone acetate inhibits spontaneous replacement of hair in intact rats, whereas intramuscular treatment with 20 μg of thyroxine accelerates spontaneous replacement; neither hormone has an appreciable effect on growth induced by plucking. If intact rats are treated with both thyroxine and cortisone, however, spontaneous growth is neither accelerated nor inhibited, and resembles the spontaneous growth of animals not treated with any hormone Induced growth is normal in these animals, and the quality of the pelage is not affected Thus the inhibitory effects of cortisone are offset by the stimulating effects of thyroxine in intact rats. Conversely, the accelerating effect of adrenalectomy on spontaneous replacement of hair is minimized when the level of thyroid hormone is decreased.

When PTU-treated animals are injected with 1 mg of cortisone daily for 4 weeks, practically all hair growth is inhibited (Figs 11 and 12). Isolated hairs occasionally grow on the plucked side at a normal rate. The skin remains extremely thin and pale during the cortisone treatment. When treatment is continued for another month, hair growth continues to be inhibited on both sides of the animal. If, however, cortisone is discontinued after 4 weeks, follicular activity can be detected on the plucked side within 1 week, after 4 weeks the coat has fully regrown on the plucked side Spontaneous growth is minimal, even after cortisone injections have been discontinued Similar results are obtained when PTU is discontinued instead of cortisone and the animals are subsequently given 10 μg of thyroxine If both PTU and cortisone are discontinued and thyroxine is given to these animals, both sides grow a full coat of hair within 4 weeks

Combined treatment with 10 μg of thyroxine and 1 mg of cortisone daily produces good regrowth of hair after plucking in rats maintained on PTU Although no activity is observed 1 week after plucking, patches of short hairs are present a week later. After 4 weeks the plucked side is almost completely covered with fully grown hair. Spontaneous growth, however, is inhibited during the entire 4 weeks of treatment

Similar results are obtained when cortisone or thyroxine and cortisone are administered to adrenalectomized-gonadectomized rats receiving PTU Hair growth is almost completely inhibited by daily treatment

with 1 mg of cortisone. After 4 weeks a few isolated hairs are present on the plucked side, but the rest of the skin is thin and devoid of hair (Fig. 13). Rats receiving this treatment gain no weight and may even lose some although they are active and appear healthy. If, in addition to cortisone, the animals are given 20 μg of thyroxine daily, induced growth is normal except for an initial delay. After 2 weeks the hairs have grown well above the surface of the epidermis and are fully grown 2 weeks later (Fig. 13). Spontaneous growth, however, is inhibited during the 4 weeks of treatment.

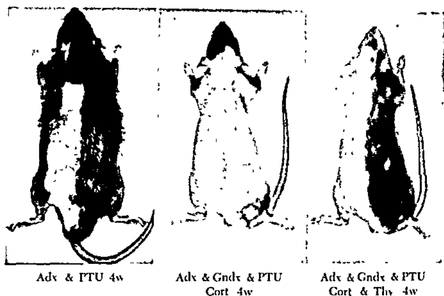


FIG 13 At right, two PTU-treated rats, 4 weeks after adrenalectomy-gonadectomy (Adx & Gndx) One milligram of cortisone acetate injected daily prevents all hair growth, while treatment with 20 μg of thyroxine (Thy) in addition to cortisone results in regrowth after plucking

At left, PTU has reduced the amount of spontaneous replacement in an adrenalectomized male

In summary, thyroxine and cortisone have opposite effects on hair growth in the rat, and one hormone counteracts the effects of the other Excess cortisone coupled with a lack of thyroid hormone inhibits hair growth profoundly. Conversely, thyroxine treatments in adrenalectomized animals stimulate the follicles of rats (Butcher, 1937).

2 *Thyroxine and Epinephrine*

Although epinephrine produces calorigenic effects only if thyroid hormone is present (Swanson, 1956), its effects on hair growth are not

mediated or potentiated by thyroxine (Table IV). Epinephrine produces its typical effects with or without the presence of thyroid hormone When PTU-treated rats are injected twice each day with 0.1 mg of epinephrine, there is a greater delay in response to plucking than in rats treated with PTU alone, but after 4 weeks growth is nearly complete. As in control animals, spontaneous replacement of hair is inhibited in the animals receiving epinephrine. If this hormone is frequently injected into the same site, induced growth becomes retarded locally and several white hairs are present at the site after 4 weeks of treatment. If, in addition to epinephrine, 1 mg of cortisone is given three times each week to hypothyroid animals, induced growth is more markedly delayed than in animals receiving epinephrine or cortisone alone. Once sufficiently developed, however, the follicles grow normally. One milligram of cortisone injected every other day does not suppress induced hair growth in PTU-treated animals.

Daily treatment with 10 μg of thyroxine produces an experimentally controlled level of thyroid hormone in PTU-treated rats. If epinephrine is administered to such rats, hair growth resembles that of normal rats treated with epinephrine, spontaneous replacement is inhibited, but regrowth of hair after plucking is normal except for an initial delay. Also, local effects are observed near the site of injection, and white hairs are eventually produced Combined treatment with epinephrine and cortisone produces effects similar to those just described, but they are more pronounced.

VI. The Effects of Diabetes and Insulin on Hair Growth

Few studies have been made of skin and hair growth in diabetic, insulin-treated, or glucose-treated animals Glucose has a marked effect on epidermal mitotic activity (Bullough, 1950a, b, 1954, Bullough and Johnson, 1951). Changes in the levels of insulin and glucose in the blood should modify hair growth, dietary caloric restriction, for example, inhibits hair growth in rats and mice (Butcher, 1939, Kozam, 1952, Loewenthal and Montagna, 1955)

A Alloxan Diabetes

Nonfasted rats were given a single intraperitoneal injection of 30 mg of alloxan monohydrate[12] per 100 grams of body weight at least 2 days before they were used for study This dose was fatal to some animals, but the survivors developed symptoms typical of alloxan diabetes.

Except for an initial delay, induced growth is normal in 4- or 8-week-old rats treated with alloxan (Table VI). Four weeks after pluck-

[12] Eastman Kodak Company

TABLE VI

EFFECTS OF DIABETES, INSULIN, AND GLUCOSE ON HAIR GROWTH

Treatment	Spontaneous waves: Frequency	Spontaneous waves: Rate of spread	Spontaneous waves: Growth within waves	Regrowth after plucking: Initial response	Regrowth after plucking: Remainder of cycle	Remarks[a]
Alloxan	Retarded	Retarded	Normal	Retarded	Normal	
Alloxan + *cortisone* (3 mg/week)	*Prevented*	Prevented	Normal	Prevented	Prevented	Cortisone fatal to alloxan-diabetic rats *No* growth present after 2 weeks
Alloxan + insulin	Normal	Normal	Normal	Normal	Normal	Insulin (2 U/d) prevents effects of alloxan Insulin (7 U/d) fatal to intact rats but skin appears in better condition than *normal and* hair seems to grow well
Phlorhizin	Normal	Normal	Normal	Normal	Normal	Phlorhizin and oil tend to accumulate under the skin After 2 weeks, rats not consistently diabetic
Glucose	Retarded	*Retarded*	Normal	Normal	Normal	Effects not found in all rats Inhibition attributed to "stress"

[a] Texture of hair normal for all treatments

ing, the hairs are fully grown (Figs. 14 and 15), but spontaneous growth is markedly retarded. Older animals have no spontaneous replacement after 4 weeks (Fig. 15), whereas younger animals have less growth than do normal animals or diabetic animals receiving insulin treatments (Fig 14). The significance of this will be discussed later.

B. *Phlorhizin Diabetes*

Eight-week-old rats become diabetic for a minimum of 2 weeks when injected subcutaneously each day with 10 mg of phlorhizin[13] suspended in oil per 100 grams of body weight. During the third and fourth weeks of treatment, however, phlorhizin and oil frequently accumulate beneath the skin and the rats are not consistently diabetic.

Hair growth initiated by plucking phlorhizin-diabetic rats is normal (Table VI), growth commences at the same time as in untreated animals, and the hairs are fully grown 4 weeks after plucking (Fig. 15) The texture of the pelage is normal and spontaneous growth waves resembled those of control rats.

C. *Glucose*

Injections of 1 ml of 10% glucose three times each day into the peritoneal cavity of intact rats produced inconsistent effects on hair growth (Table VI). Spontaneous replacement was greatly retarded in some rats, less so in others (Fig 15). Regrowth of plucked hairs, on the other hand, was normal in all animals Similar effects on hair growth were observed in rats given a drinking solution containing 50% glucose

D. *Insulin*

Daily treatment with two units of protamine-zinc insulin[14] restores spontaneous replacement to normal in alloxan-diabetic animals (Fig 14) Although there is no longer an initial delay in follicular activity after plucking, the cycle of growth remains normal (Table VI). Daily injection of 7 units of insulin proves fatal to intact rats, however, a few animals have survived more than 2 weeks of treatment. Induced growth appears to be normal despite the poor health of these animals There is some spontaneous growth in the rats receiving insulin, but reliable observations could not be made on the spread of activity.

E *Analysis of the Effects of Glucose and Insulin*

It is difficult to distinguish the effects of insulin from those of blood glucose on hair growth, since these two factors are intimately related

[13] Amend Drug and Chemical Company

[14] Eli Lilly and Company

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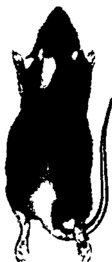


Allox. 2w









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Allox 4w

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FIG 15 Hair growth in 8-week-old rats treated with alloxan (Allox), phlorhizin (Phlor.), or glucose for four weeks Spontaneous growth was inhibited by alloxan-diabetes but was not affected by phlorhizin treatment Glucose injections often caused a delay in spontaneous replacement, as illustrated Induced growth was normal in all these animals except for an initial delay in the alloxan-diabetic rat.

An attempt has been made to overcome this obstacle by observing hair growth in rats treated with alloxan, phlorhizin, insulin, and glucose Alloxan-diabetic rats have no source of insulin, and their level of blood glucose is elevated. Phlorhizin-treated animals have a low level of blood glucose, and their insulin production is minimal Insulin injections raise the circulating level of this hormone but automatically lower the blood glucose level, whereas glucose injections raise the concentration of glucose in the blood and stimulate insulin production Comparison of the results obtained from each treatment should reveal the separate effects of insulin and glucose on hair growth (Table VI).

Except for an initial delay in alloxan-treated animals, regrowth after plucking is normal in both alloxan- and phlorhizin-diabetic rats, and the texture of the pelage is not affected. Spontaneous replacement is markedly retarded after alloxan treatment, but is normal in rats receiving phlorhizin. Insulin, then, is needed more critically for the early stages of hair growth than is glucose This conclusion is supported by the following facts· (a) Insulin treatments abolish the inhibitory effects of alloxan on hair growth. (b) Injections of insulin in normal rats maintain growth, despite the low level of circulating glucose. (c) Growth after plucking is normal in glucose-treated animals, whereas spontaneous replacement is often inhibited This inhibitory effect of glucose probably represents a generalized "stress reaction," since similar effects are obtained when either epinephrine or cortisone is administered to intact rats. Preliminary observations of cortisone-treated, alloxan-diabetic rats show a pronounced inhibition of hair growth.

Insulin and not glucose seems to be the critical factor in the growth of hair follicles. Unlike the epidermis (Bullough, 1950a, b, Bullough and Johnson, 1951), hair follicles are able to grow normally despite changes in the concentration of blood glucose Although both oxygen and a carbohydrate source are essential for maintaining the mitotic activity of a hair follicle *in vitro*, drastic conditions such as starvation or shock are required to influence such activity *in vivo* (Bullough and Laurence, 1958) The abundant supply of glycogen in growing hair follicles may partially account for the relative independence of the hair follicle from the level of circulating glucose (Johnson and Bevelander, 1946, Montagna *et al*, 1951, 1952)

Insulin has a mitogenic effect on the epidermis, provided that glucose is present, by accelerating the hexokinase reaction (Bullough, 1954) Some such mechanism may be involved in the enhancement by insulin of the initiation and the early stages of hair growth This may also explain the decrease in chondroitin sulfuric acid and hyaluronic acid in the skin of alloxan-diabetic rats (Schiller and Dorfman, 1955)

VII. The Effects of Pituitary Hormones on Hair Growth

A. *Hypophysectomy*

Investigators do not agree on the effects of hypophysectomy on hair growth (Baker, 1951, Cooper, 1930, Houssay *et al*, 1955). Early studies on the effects of hypophysectomy in dogs reported inhibition of hair growth (Houssay, 1918). Later, Freud (1934) reported that plucked hairs regrow slowly in hypophysectomized rats and that treatment with growth hormone prevents this retardation. The results of these workers are difficult to assess, since their methods of study are not clearly described. Snow and Whitehead (1935) found that hypophysectomy decreases the rate of spontaneous replacement and that treatment with a growth hormone preparation restores the pilary system to its normal state. In their investigations, however, little or no attention was paid to the periodic waves of spontaneous hair growth nor to the age of the animals Dieke (1948), who was familiar with the periodicity of spontaneous growth waves, also found an inhibition of hair growth after hypophysectomy Hundley and Ing (1951), in contrast, reported an immediate initiation of hair growth after hypophysectomy. Others have also observed that hypophysectomized rats grow a considerable amount of hair, probably caused by adrenal atrophy (Baker, 1951, Houssay *et al*, 1955).

Under scrupulous experimental conditions, hypophysectomy accelerates the initiation of spontaneous growth (Table VII) Follicular activity develops immediately over the entire clipped side One week after the operation the skin of the entire side is uniformly colored, whereas in sham-operated animals only the belly skin is colored After 2 weeks the follicles are actively producing hair (Fig 16), and hair growth is completed after 4 weeks except near the tail (Fig 17) If the entire back of these animals is clipped 1 month after hypophysectomy, a full coat of soft hair grows back within the next 4 weeks. Induced growth is normal, but the hair which grows after hypophysectomy is very soft and resembles that of immature animals

Acceleration of hair growth after hypophysectomy is attributed to atrophic changes in the adrenal cortex (Baker, 1951), which commence at once after removal of the pituitary (Deane and Greep, 1946). The pituitary exerts a restraint on hair growth probably by way of the adrenal cortex and hypophysectomy removes this restraint. In rats hypophysectomized for a long period of time there may be a decrease in hair growth, resulting from the loss of appetite, poor physiological and metabolic conditions. and atrophy of the remaining endocrine glands, especially the thyroid gland

TABLE VII

HAIR GROWTH IN RELATION TO PITUITARY HORMONES

Treatment	Spontaneous waves			Regrowth after plucking		Hair texture	Remarks
	Frequency	Rate of spread	Growth *within* waves	*Initial* response	Remainder *of* cycle		
Hypophysectomy	Accelerated	Accelerated	Normal	Normal	Normal	Infantile	Subsequent waves also accelerated, but induced growth normal No change in pattern of waves Effects attributed to lessened *adrenocortical secretion* Decreased thyroid activity may later affect growth
Hypx + cortisone (1 mg/d)	Prevented	Prevented	Normal	Prevented	Prevented[a]	Infantile	Cortisone prevents all growth except in existent growing follicles Effects may be related to decreased thyroid secretion
Hypx + estrogen	Retarded	Retarded	Retarded	Retarded	Retarded	?	Rats die within 2 weeks, but inhibition very apparent
Hypx + androgen	Accelerated	Accelerated	Normal	Normal	Normal	Infantile	Androgen without effect in hypx. rats Acceleration due to hypophysectomy
Hypx. + chorionic gonadotropin	Retarded	Retarded					Houssay *et al.* (1955)

TABLE VII (*Continued*)

Treatment	Spontaneous waves			Regrowth after plucking		Hair texture	Remarks
	Frequency	Rate of spread	Growth within waves	Initial response	Remainder of cycle		
Growth hormone	Normal	Normal	Normal	Normal	Normal	Normal (adult in hypx.)	No effect in intact adults, but may enhance waves in young rats Produces an adult pelage in hypx. rats (Smith, 1930, Snow and Whitehead, 1935) but has no effect on waves (Houssay *et al*, 1955)
Adrenocorticotropin	Retarded	Retarded	Normal	Retarded	Normal	Normal (infantile in hypx)	Retards waves in intact and hypx rats Has no effect after adx Hair remains infantile after hypx No pigmentary changes
Luteotropin	Normal	Normal	Normal	Normal	Normal	Normal	No effect in normal females but may maintain inhibition in females separated from their young

[a] See Table VIII

B. *Growth Hormone (Somatotropin)*

The hair of hypophysectomized animals is fine and infantile (Smith, 1930), and pituitary implants, or injections of growth hormone restore an adult pelage in hypophysectomized rats (Smith, 1930, Snow and Whitehead, 1935, Thompson and Gaiser, 1932). Changes in the quality of the pelage after hypophysectomy and the prevention of these changes by the administration of the growth hormone are probably not due to nutritional factors The animals used in the present studies developed a

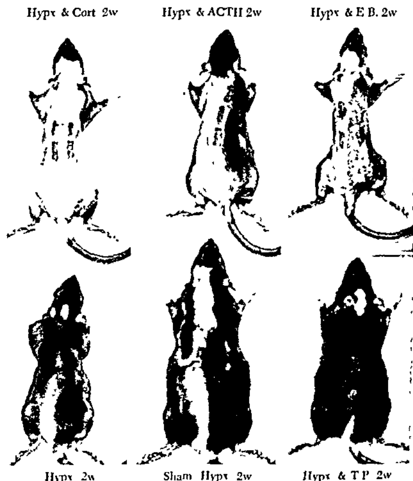


FIG 16 Rats hypophysectomized (Hypx) for 2 weeks. Compare the inhibitory effects of 1 mg of cortisone acetate with those of 250 µg of ACTH Estrogen has markedly inhibited growth, whereas androgen has not Spontaneous growth in the untreated, hypophysectomized rat is clearly more advanced than that in the sham-operated animal

juvenile coat after hypophysectomy even with milk added to the diet. Furthermore, somatotropin (STH) does not appear to produce its effects on hair quality by influencing other endocrine glands. Growth hormone, itself, seems necessary for the production of an adult pelage.

The effects of growth hormone on the growth of hair are not clear. Although Freud (1934) and Snow and Whitehead (1935) reported that growth hormone enhances the growth of hair in hypophysectomized











FIG 17 The same hypophysectomized (Hypx) rats shown in Fig 16, 2 weeks later There is still no trace of hair in the cortisone-treated animal Testosterone (T P) has no effect on the quality of the fur Estradiol benzoate is fatal to these animals

rats, Emmens (1942) found that of several preparations of anterior pituitary extracts only one affected the rate of spontaneous replacement in normal female rats. Also, other investigators (Butcher, 1937; Houssay *et al*, 1955; Scow *et al.*, 1949) have reported that growth hormone has *no effect on hair growth in intact, adrenalectomized, thyroidectomized,* or hypophysectomized rats. Interestingly, somatotropin depresses epidermal mitotic activity *in vitro* (Bullough, 1954).

In the present studies daily treatment with 100 μg of growth hormone[15] given intramuscularly had no effect on hair growth in 14-week-old rats, although these animals usually gained more weight than did the untreated control animals. Spontaneous growth waves spread as rapidly in the control rats as in rats receiving growth hormone, and induced growth was similar in the two groups of animals (Table VII). In 4-week-old rats, however, there appeared to be a tendency toward more rapid replacement of hair when the growth hormone was given

C. *Adrenocorticotropin*

Adrenocorticotropin (ACTH) inhibits hair growth in intact and gonadectomized rats (Asling *et al*, 1951, Baker, 1951, Baker *et al*, 1948, Moon, 1937). (See Table VII) Daily intramuscular treatment with 250 μg of ACTH[16] produces effects in intact rats similar to those produced by cortisone Spontaneous waves of growth are retarded, but once the follicles become active they grow at a normal rate.

The pelage that grows during treatment with ACTH is infantile in hypophysectomized rats. Growth induced by plucking immediately after the operation is retarded One week after hypophysectomy the skin of ACTH-treated animals shows only traces of color, but there is abundant color in the skin of untreated rats. After 2 weeks, hair is not as long as that of control rats (Fig 16), and after 4 weeks the hair follicles are just finishing their growth (Fig 17) ACTH also retards spontaneous growth One week after hypophysectomy no activity is evident, after 2 weeks only slight activity is discernible on the belly (Fig 16), after 4 weeks the follicles are still growing, especially in the sacral region (Fig 17) If the ACTH treatment is discontinued after 4 weeks, one can see by clipping the entire back that a completely new coat of hair grows on both sides within 4 more weeks Other investigators (Asling *et al*, 1951, Houssay *et al*, 1955) have also reported such inhibition of spontaneous growth

The inhibitory effects of ACTH on hair growth are mediated through the adrenal cortex since ACTH has no effect on either spontaneous or

[15] Armour and Company
[16] National Drug Company

induced hair growth in adrenalectomized rats (Table VII) Spontaneous replacement continues to be accelerated, as in adrenalectomized rats not treated with ACTH, and follicular activity initiated by plucking proceeds at the normal rate (Figs. 4 and 5).

ACTH preparations often contain intermedin (melanocyte-stimulating hormone, MSH), which may produce changes in pigmentation in the skin (Hall *et al*, 1953; Lerner *et al*, 1954, Sulman, 1952). However, no changes in pigmentation have been observed in intact, adrenalectomized, or hypophysectomized rats, whether receiving ACTH or not (Figs 4, 5, 16, and 17).

D. *Thyrotropin*

Hypophysectomized rats treated with cortisone are strikingly similar to those receiving cortisone and PTU (Tables V and VII). Hypophysectomized rats treated intramuscularly with 1 mg of cortisone acetate each day for 1 month have practically no hair growth (Figs. 16 and 17). A few hairs emerge on the belly from follicles which were growing at the start of the experiment. These hairs grow at a normal rate. The rest of the skin remains bare and is very thin. When treatment is continued for another month, cortisone continues to prevent hair growth. If, however, cortisone treatment is stopped after 1 month, follicular activity is seen on the plucked side within 1 week, and by 4 weeks a completely new coat of juvenile hair has grown.

Since similar results are obtained if cortisone is given to thyroid hormone-deficient rats, the great inhibition of growth produced by cortisone in hypophysectomized animals probably reflects the diminished activity of the thyroid gland which follows hypophysectomy. The pituitary gland not only tends to inhibit hair growth by the production of ACTH, but also enhances growth by producing thyrotropin (TSH).

E. *Gonadotropins*

Chorionic gonadotropin is said to inhibit hair growth in hypophysectomized rats (Houssay *et al*, 1955) The results of experiments with gonadal hormones in hypophysectomized animals indicate that gonadotropin, through its effects on the gonad, may control the texture of the pelage only if an additional pituitary factor is present

Estradiol benzoate is very toxic to hypophysectomized animals, and none of the animals which received 50 µg of this substance intramuscularly each day survived longer than 15 days After 1 week of treatment, traces of pigmentation are seen only on the plucked side, after 2 weeks, hair has just emerged on the plucked side, and on the clipped side a moderately colored band extends along the body from the belly

halfway to the dorsal midline (Fig 16). Hypophysectomized littermates not receiving estrogen show abundant growth on both sides 2 weeks after hypophysectomy. Estrogen, then, inhibits both spontaneous and induced hair growth in hypophysectomized rats (Table VII).

In contrast, androgen has no effect on hair growth in hypophysectomized animals (Tables III and VII); the skin on the plucked side of rats treated daily with 200 μg of testosterone propionate is uniformly colored after 1 week, and the hair follicles are actively growing after 2 weeks (Fig 16). Growth is completed within 4 weeks, but the pelage is infantile (Fig 17). Spontaneous growth is also unaffected by treatment with testosterone, and growth is nearly complete after 4 weeks of treatment.

These results suggest that gonadal and gonadotropic hormones modify the type of hair produced only if some pituitary factor is present. Presumably, this pituitary factor is growth hormone since it does affect the quality of the pelage. Estrogen inhibits hair growth even in the absence of the pituitary.

VIII. The Effects of Selected Hormonal Imbalances on Follicles in Different Stages of Growth

The concluding experiments of this study were designed to test whether or not hair follicles in different stages of activity are equally affected by experimentally imposed endocrine imbalances One experimental group consisted of 10 animals that had been receiving PTU for several weeks A second group consisted of 7 4-week-old and 14 8-week-old animals which had received no previous treatment. The back of each rat in both groups was prepared for study in the following manner the posterior right quarter was plucked 6 days before the day of the experiment, the posterior left quarter, 4 days before, the anterior right quarter, 2 days before, and the anterior left quarter, the day the experiment began. Those follicles plucked 4 or 6 days earlier would be fairly well differentiated and would be commencing to produce hair, whereas those plucked on the day the experiment began, or 2 days before would show only the earlier follicular differentiation

Of the 10 rats receiving PTU, 4 were given 1 mg of cortisone acetate intramuscularly in saline each day, 3, 1 mg of cortisone and 20 μg of thyroxine, also in saline, and 3 received nothing Twelve animals from the second group were hypophysectomized, 7 of these received 1 mg of cortisone daily, the other 5 received no cortisone. Of the 9 intact animals now remaining in the second group, 5 received 10 mg of cortisone each day and 4 were left untreated. Each area was studied at weekly intervals from the time it was first plucked until 4 weeks after

the injections were begun. The results of these experiments are summarized in Table VIII and are illustrated in Figs. 18 and 19

In animals made thyroid hormone-deficient with PTU, daily treatment with 1 mg of cortisone completely inhibited growth in areas plucked on the same day cortisone treatment began, or 2 days before it began (Fig. 18). Hair growth is normal in the area plucked 4 days before initiation of cortisone injections but the hairs are sparse, there is abundant growth in follicles plucked 6 days before the starting of the experiment PTU-treated animals which received no cortisone show essentially normal induced growth in all four quarters. The same is true of animals which received 20 μg of thyroxin per day in addition to cortisone (Fig. 18)

Intact rats treated daily with 10 mg of cortisone show a marked inhibition of hair growth (Fig. 19). No hair grows in the areas plucked on the day the treatments were started or 2 days before, sparse growth appears near the belly in the area plucked 4 days before the beginning of the experiments and in that plucked 6 days before, and proceeds normally. Young rats, 4 weeks of age, die during this treatment.

When hypophysectomized animals are treated daily with 1 mg of cortisone there is no hair growth in areas plucked on the day of the operation or 2 days before (Fig. 19). The cycle of hair growth is normal in the area plucked 4 days before hypophysectomy, but not all follicles grow. In the area plucked 6 days before the operation growth is normal All hair growth is infantile in hypophysectomized animals. Induced growth is normal regardless of the stage of activity at the time of hypophysectomy provided no hormones are injected, and the hair is infantile (Fig. 19).

IX Summary

The growth of hair is highly resistant to endocrine disorders but an intricate balance of many hormones is required for the normal occurrence of the periodic waves of spontaneous replacement (Fig 20) Hormonal imbalances upset the normal periodicity of spontaneous replacement in rats. The exact nature of each hormonal imbalance determines whether or not follicles become active, but, except for estrogen, hormones have little effect on the cycle of growth once it has been initiated. Estrogen prolongs the entire period during which existent hairs grow Some hormones also seem to control the type of hair produced.

In the normal rat there are alternate periods of activity and rest in the hair follicles. Follicular activity begins spontaneously on the belly and spreads dorsally in a wavelike fashion. As the animal grows older the waves of replacement are less frequent and spread more slowly

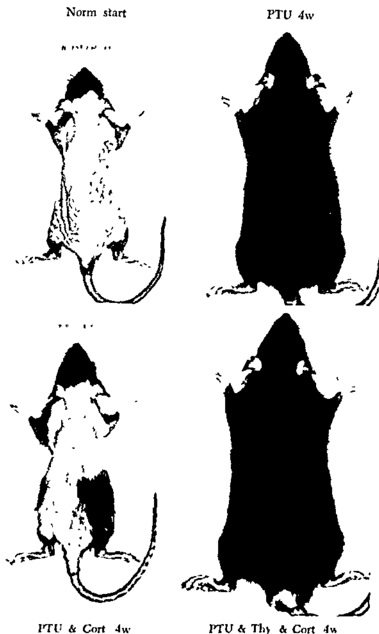


FIG 18 The effects of various hormonal states on hair follicles in different stages of growth When the experiment started, the upper right quadrant had been plucked for 2 days and the upper left had just been plucked The lower right and left quadrants had been plucked 6 and 4 days earlier, respectively Observe the appearance of an animal at the beginning of the experiment, then compare the amount of regrowth in the four quadrants of each animal 1 month later









FIG 19 Results of an experiment similar to that in Fig 18, in which additional hormonal states were used Compare the effects of daily treatment with 1 mg of cortisone acetate in the hypophysectomized (Hypx) rat with its effects in the PTU-treated rat shown in Fig 18

TABLE VIII

RESPONSE OF HAIR FOLLICLES IN DIFFERENT STAGES OF GROWTH TO TREATMENTS PRODUCING SPECIFIC HORMONAL STATES

Treatment	Response of follicles to 4 weeks of treatment: Stage of Growth at Start (No. days after plucking) 0	2	4	6
Normal	Normal	Normal	Normal	Normal
Cortisone (10 mg/d)	No response	No response	Normal (sparse growth)	Normal (sparse growth)
PTU	Normal but delayed	Normal but delayed	Normal	Normal
PTU + cortisone (1 mg/d)	No response	No response	Normal (sparse growth)	Normal
PTU + cortisone (1 mg/d) + thyroxine (20 µg/d)	Normal	Normal	Normal	Normal
Hypophysectomy	Normal[a]	Normal[a]	Normal[a]	Normal[a]
Hypx + cortisone (1 mg/d)	No response	No response	Normal[a] (sparse growth)	Normal[a]

[a] Infantile hair

Male rats have coarse hair, and their skin is covered with flakes of oxidized lipid The pelage of females is finer, the skin has no lipoid scales, and the occurrence of spontaneous growth waves tends to lag behind that in males. After gonadectomy the texture of the fur is intermediate between that of males and females, and spontaneous waves resemble those of males. Testicular hormone then, promotes coarseness of pelage and ovarian hormones prevent it, and retard the waves of hair growth The successive phases in a particular follicle during one cycle of growth are the same in both sexes.

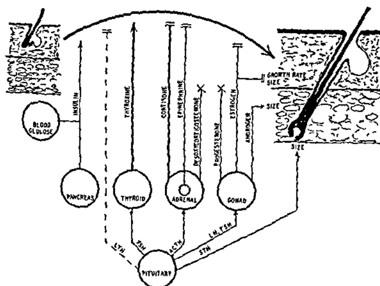


FIG. 20 Schematic representation of the effects of hormones on hair growth The spontaneous conversion of resting follicles into growing ones is profoundly influenced by several hormones, as illustrated. In growing follicles, androgen merely promotes a coarse pelage, whereas estrogen prolongs the entire period of growth and prevents the development of coarse hair Growth hormone is necessary for an adult coat. KEY ⟶ Stimulates, ——| | Inhibits, ——× No effect, — — — — Effect uncertain

Daily treatment with estrogen retards the initiation and the rate of both spontaneous and induced hair growth in normal, gonadectomized, and thyroid hormone-deficient rats The inhibition by estrogens is less apparent after adrenalectomy or hypophysectomy, since removal of these glands accelerates growth. Estrogen causes the growth of fine, sparse hair in all animals except in those which have been hypophysectomized, and produces some of its effects independent of the adrenal cortex. Daily treatment with androgen has no apparent effect on hair

growth except that it produces a coarse pelage in all except the hypophysectomized rat.

During pregnancy and lactation spontaneous replacement of hair is noticeably retarded, but spontaneous growth is transiently accelerated when the young are removed from the mother. The time required for replacement of plucked hairs is normal in these animals. Endogenous estrogen may be involved in the retardation found in pregnant animals, whereas luteotropin may inhibit replacement during lactation. Progesterone appears to have little effect on hair growth.

Adrenalectomy accelerates the initiation and the spread of spontaneous follicular activity, but it does not affect the rate of growth of the individual follicle. Conversely, daily treatment with small doses of cortisone inhibits the spontaneous initiation of growth in intact, gonadectomized, or adrenalectomized rats. Once growth has started, however, cortisone has no apparent effect There is no cumulative effect after long periods of treatment with cortisone, and the follicles do not become "refractory" to the hormone. Large doses of cortisone inhibit hair replacement in intact rats even after plucking, except in follicles which were plucked 4 to 6 days before the treatment started Similar effects are obtained when small doses of cortisone are given to PTU-treated rats or to hypophysectomized ones In all these cases growth commences as soon as the cortisone is discontinued. Since daily treatment with deoxycorticosterone has no detectable effect on hair growth in intact or adrenalectomized rats, it appears that the adrenal cortex normally restrains the initiation of spontaneous growth by means of its glucocorticoids

When rats are made deficient in thyroid hormone through treatment with PTU, spontaneous replacement of hair is prevented for a considerable period of time, but induced growth is relatively normal. Conversely, thyroxine accelerates spontaneous replacement of hair in PTU-treated rats and in normal ones, but the cycle of growth is normal whether induced or spontaneous The thyroid gland must normally exert a stimulating effect on the hair follicles which balances the inhibitory effect of the adrenal cortex Thyroxine and cortisone have antagonistic effects, and one hormone can be used to offset the effects of the other There is no such relationship between thyroxine and gonadal hormones

Continuous treatment with epinephrine inhibits spontaneous hair growth in intact animals and delays the response to plucking, but once growth has started it proceeds normally. Prolonged treatment with epinephrine produces a local inhibition of spontaneous and induced growth Growth waves tend to by-pass the area of injection, and induced

growth is retarded there. The hairs which eventually grow near the sites of injection have no pigment. The local effects of epinephrine may be due in part to changes in the vascular system, while the general effects may be mediated through the adrenal cortex. Thyroid hormone does not appear to be involved in any of the effects of epinephrine on hair growth.

Spontaneous replacement is markedly retarded in alloxan-diabetic animals, but induced growth is normal after an initial delay. Phlorhizin treatment does not affect growth, even though there is continued glycosuria and hypoglycemia. Insulin restores spontaneous replacement to normal in alloxan-diabetic animals and tends to enhance growth in intact animals, despite the low blood glucose level. Glucose-treated animals display normal regrowth after plucking, and spontaneous growth is often retarded. Insulin seems to be more directly involved in hair growth than glucose, provided that sufficient glucose is present to maintain growth.

Hypophysectomy accelerates the initiation and spread of spontaneous follicular activity. The cycle of growth is normal after plucking, but the pelage is infantile. The administration of ACTH has an inhibitory effect on hair growth in intact and hypophysectomized animals, but it is without effect in adrenalectomized animals. The pituitary, then, tends to inhibit hair growth in the normal animal by means of the adrenal cortex. However, it probably stimulates hair growth through the thyroid gland. Growth hormone restores the pelage of hypophysectomized rats to an adult texture, but gonadal hormones do not. Sex hormones modify the hair produced only if growth hormone is present. Aside from this, growth hormone appears to have little effect on hair growth.

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CHAPTER 16

Age, Sex, and Genetic Factors in the Regulation of Hair Growth in Man: A Comparison of Caucasian and Japanese Populations[1]

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[1] This analysis is based upon detailed studies made in conjunction with Gordon E Mestler, Harumi Terada, Leitha Bunch, and Eiji Inouye Data for individual subjects with more complete statistical treatment are presented separately for each of the three large groups of subjects intact Japanese (Hamilton *et al*, 1958), intact Caucasians, and Caucasian eunuchs

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I. Introduction

Methods have been formulated to permit quantitative measurements of hair in circumscribed regions in man and studies have been made of each sex at successive ages. From data for approximately 3000 subjects (*Hamilton, 1951a, Hamilton et al., 1955, 1958) standards have been* constructed for use in studies of endocrine status and assessment of some aspects of physiologic age.

Attention is paid to the actions and interrelationships of endocrine, ageing, genetic, and nutritional factors As part of an attempt to distinguish between genetic and environmental influences, Caucasian and Mongolian populations were compared since these two groups tend toward somewhat polar extremes in the degree of development of many secondary sex characters, including certain pilary areas. In a further effort to separate genetic and environmental factors, investigations were made of twins and families, and of the effects of environment as indicated by comparison of subjects of Japanese ancestry living in Tokyo vs New York City.

A primary aim of this work was the quantitative measurement of certain aspects of maleness, in the hope of furthering the understanding of factors responsible for the predilection of males (in comparison with females) for a large number of pathologic states and for a substantially shorter duration of life. The nature and significance of the poor viability association with maleness in man and many other species has been examined elsewhere (Hamilton, 1948, Hamilton *et al*, 1958), here it suffices to note the importance of this lesser viability of the male which represents, in its totality, a considerably greater loss of potential life than that from any specific disease except heart diseases, which is itself a condition that occurs earlier and more frequently in males than in females

Since the methods employed have not been published in detail, mention need be made of variables encountered, potential errors, and reliability of these procedures Some of the techniques are based upon weight of hair grown per 24 hours, as calculated from shavings of the beard at intervals of 48 hours and of the axilla at intervals of 42 days, the procurement of reliable data with these procedures requires elimination of diurnal variation and the minimization of errors due to hygroscopic properties of hairs and to contamination by lipids and epithelial debris. Detailed studies have shown that no important errors are introduced by shaving or by environmental changes, at least in adults in the north temperate zone of the United States, in the adolescents under investigation we have avoided the possibility of seasonal variation. Methods, other than those based on the weight of timed and specially

processed collections, include measurements of the areas with coarse hairs, the total number of coarse hairs and the number per square centimeter in a standardized site, the percentage of gray hairs, and the microscopic study of shavings after most downy hairs and debris have been removed in processing the sample.

The microscopic measurements of beard hairs were made at the base of cut hairs and are reported as mean values per subject, as determined for 15 randomly selected hairs studied as to breadth of (a) the hair, (b) its medulla, and (c) its cortex plus cuticle.

Measurements of axillary hairs, recorded as mean values per subject, were made of the lengths of the 3 longest hairs in the fully grown mass and the breadths of these hairs at their bases.

The percentage of gray hairs was determined from examination of approximately 160 hairs randomly selected from beard shavings and from inspection of all hairs in axillary shavings.

The procedures employed were chosen after pilot studies and provide closely comparable data in duplicate analyses. In serial studies of the same subjects, the degree of agreement was excellent for the various measurements of beard, the coefficient of variation was as low as 7 in measurements of the area covered by coarse hairs and only as high as 15 in random sampling of breadth of hairs collected from the entire bearded area of face and neck The variation in serial studies of axillary hair in the same subject tended to be greatest in data for weight of hair grown per day, fluctuations were as much as 50% in a few subjects.

This battery of procedures was designed to elucidate different facets of hair growth, and the methods are applicable whether the growth of hair be limited or extensive. The correlations of data obtained by different methods for various pilary regions and ranges of ages require more detailed analysis than can be given here

II. Results

A Beard

1. *Comparisons of Twins and Less Closely Related Males*

Studies were made (Hamilton *et al*, 1958) of 10 pairs of monozygotic (MZ) twins, of 6 pairs of dizygotic (DZ) twins, and of nontwin brothers and fathers in 24 families

Configuration of the bearded area was almost exactly alike in identical twins, seldom so between other male siblings, even in fraternal twins, and quite variable among unrelated males of the same age and ethnic group.

In weight of beard grown per day, intrapair differences were significantly less in MZ and DZ twins than in unrelated males of the same age (Hamilton *et al*, 1958). Intrapair differences between father and son, or between brothers who were not twins, were not significantly smaller than between unrelated males.

The breadth of hairs and of their component parts also tended to be much more similar in identical twins than in pairs of males of similar age who were less closely related genetically.

These data point to the importance of genetic factors in the regulation of beard growth

2. Comparisons of Caucasian and Japanese Males at Successive Ages in Randomly Selected Populations

a Weight of beard grown per 24 hours (Fig. 1). In Caucasian as compared with Japanese males, the weight of beard grown per day was considerably greater and peak values were attained earlier in life. Among females, too, facial hirsutism tended to be much less in Japanese than in Caucasians No instance of facial hirsutism was observed in a survey of 335 Japanese females, 5 to 88 years of age, which contrasts

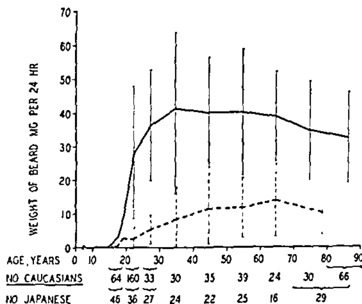


Fig 1 Mean weight of beard grown per day at successive ages by normal males in randomly selected Caucasian and Japanese populations Each vertical line represents ± 1 S D from the mean of that age group

sharply with the incidence of 10 to 28% observed in white females by Trotter and Danforth (1922), Pedersen (1942), and Dupré and Duflos (1902). Other differences, presumably genetic, have been reported previously (Trotter, 1942) in that the thickness and length of individual hairs of cheek and lip are greater in white than in Negro females.

In each age group of males the standard deviation (S D.) of values from the mean (shown graphically as vertical lines in Fig 1) indicates a large degree of variation among subjects of the same age and ethnic group. Wide variation from individual to individual is characteristic not only of the beard but of all ten secondary sex characters and traits which we have investigated for possible use as endocrine indicators. That genetic factors are responsible for much of this variation is strongly suggested by the greater concordance of values in identical twins than in fraternal twins or in less closely related males of comparable age.

Data in the following paragraphs indicate that the divergence between Caucasian and Japanese males in weight of beard grown per day arises in large part from differences in the size of area with coarse hairs and in the number of such hairs in these regions. The disparity in beard weight between these two groups of males is not attributable to differences in the breadth of mature hairs.

b. Area of face and neck with coarse beard hairs (Fig. 2), *and the number of coarse hairs per square centimeter of a standardized region of the cheek* (Fig 3). Both the area covered by coarse hairs and the number of such structures per square centimeter of a standardized region of the cheek were significantly greater ($P < 0.001$) in Caucasian than in Japanese males, as analyzed for all subjects 30 to 70 years of age

c. Breadth of coarse hairs in randomly sampled shavings of all areas with coarse beard hairs (Fig 4). The mean diameter of beard hairs, and of those component parts of the hairs which were studied, was comparable in Caucasian and Japanese males after 25 years of age Between 15 and 24 years of age the breadth of hairs was significantly greater ($P = 0.001$) in Caucasians than in Japanese, presumably associated with an earlier age of attainment of peak values for beard, as measured by various methods.

d Percentage of gray hairs (Fig 5). The incidence of this criterion of ageing among coarse hairs of the beard was significantly higher ($P < 0.001$) in Caucasians than in Japanese of all ages This was evident not only in mean values (Fig 5) but also in the medians for each decade.

Mean (Fig 5) and median values for the 70-79-year-old group of Japanese were low in comparison with those of younger and older sub-

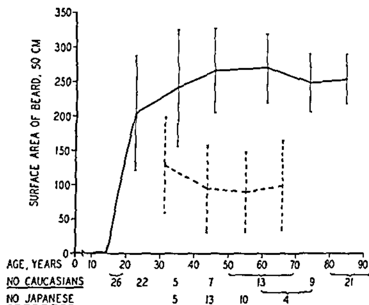


FIG 2 Mean surface area covered by coarse beard hairs in males Studies of Japanese subjects were limited to the fourth through the eighth decades

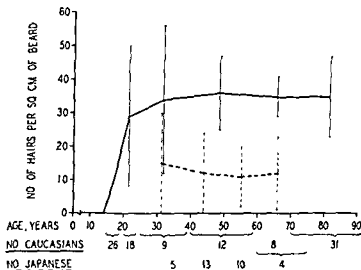


FIG 3 Mean number of coarse hairs per sq cm in a standardized area of cheek in males Studies of Japanese subjects were limited to the fourth through the eighth decades

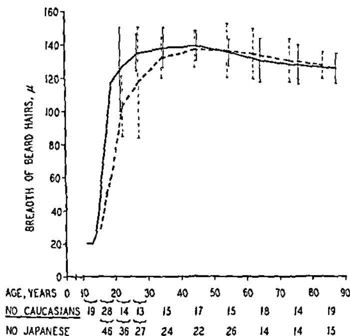


FIG 4 Mean breadth of coarse hairs selected at random from shavings of entire bearded area in males

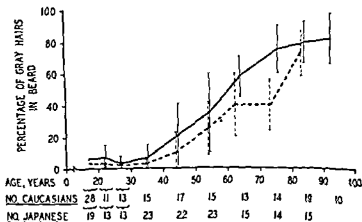


FIG. 5 Mean percentage of gray hairs in beard shavings of males.

jects, but the peculiarity of this or any one age group was not responsible for the lower incidence of grayness in Japanese than in Caucasians; in calculations that omit all subjects in the eighth decade in both ethnic groups, there was still a significantly higher incidence of grayness in Caucasian than in Japanese beards, as tested in subjects from the fourth to the ninth decades ($P < 0.001$).

B. *Axillary Hair*

1. *Comparisons of Twins and Less Closely Related Subjects*

Studies were made of 17 pairs of MZ twins, and 10 pairs of DZ twins, and members of 36 families

In general the similarity in values for weight of hairs grown per day among genetically related subjects was less for axillary hair than for beard Nonetheless, intrapair differences in measurements of axillary hairs were significantly less in identical twins than in more distantly

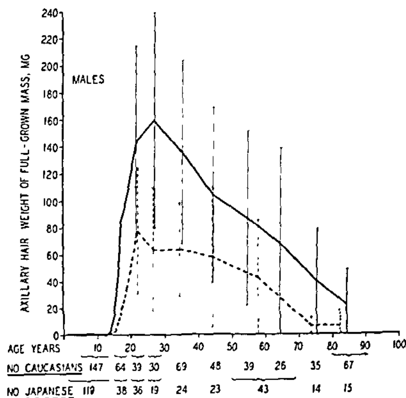


FIG 6. Mean weight of fully grown mass of hairs in right axilla of males.

related subjects with regard to configuration of the hairy area, weight of hair grown per day, and certain microscopic measurements.

2 *Comparisons of Caucasian and Japanese Males and Females at Successive Ages*[2] *in Randomly Selected Populations*

a. Weight of fully grown mass of hairs in right axilla (Figs. 6 and 7) *and milligrams of hair grown per day in right axilla* (Figs. 8 and 9).

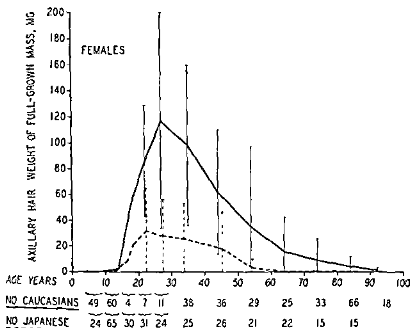


FIG 7 Mean weight of fully grown mass of hairs in right axilla of females

Data from these two types of measurement showed the same trends and are not discussed separately

Shavings were substantially heavier in Caucasians than in Japanese. The difference between these ethnic groups was more pronounced in females than in males. The ratio between the two ethnic groups at the age of peak values was 4.1 in females and 2:1 in males Growth of axil-

[2] Axillary hair is present in males and females of all ethnic groups so that its progressive and retrogressive changes may be compared in both sexes of various populations Values for the weight of hair grown per day show closely similar trends to those observed with studies of the full-grown mass of hair and may be substituted if cosmetic practices prevent study of the fully grown mass of hair.

lary hairs continued for several years after the menopause in many of the Caucasian women but in very few of the Japanese women.

As tested in males the disparity between the ethnic groups in weight of hair grown per day was not as great for axillary hair as for beard.

In both sexes the values reached a peak in the third decade and declined steadily thereafter. In females, as contrasted with males,

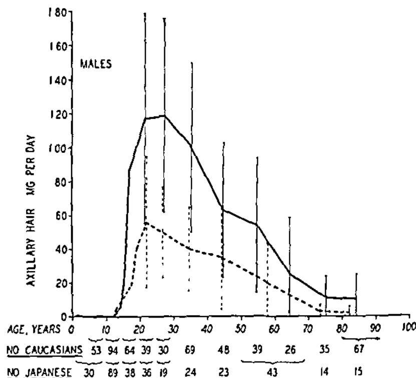


FIG 8 Mean weight of hairs grown per day in right axilla of males for whom data are presented in Fig 6

growth of axillary hairs tended to begin at a slightly earlier age (compare Figs 8 and 9), peak values were substantially less (compare Figs 6 and 7), and the decrease by the sixth decade was much more marked (compare Figs 8 and 9) Presumably, the earlier growth of these hairs in females than in males is due to earlier maturation whereas the more marked decrease in later decades in females is ascribable to the abrupt and drastic decline in gonadal secretions in the female sex

b. Number of hairs in shavings of fully grown mass of hairs in right axilla In comparison with Japanese, Caucasians had considerably larger

numbers of hairs at all ages (Figs. 10 and 11). At most ages the ratios were 2:1 or more. At the age for the menopause or later the disparity between Caucasians and Japanese was greater in females than in males. In both ethnic groups the mean values for menopausal women were considerably higher than the medians, since the presence of large numbers of hairs in a few women weighted the averages unduly.

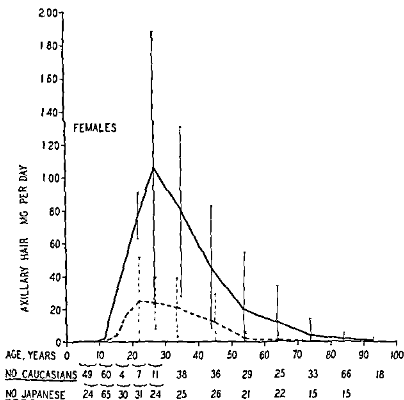


Fig 9 Mean weight of hairs grown per day in right axilla of females for whom data are presented in Fig 7

In males and females of both ethnic groups, peak values were attained in the years following sexual maturation and the number of hairs decreased progressively and substantially thereafter (Figs 10 and 11).

The age curves for number of hairs approximated those for the weight of hairs in males (compare Figs. 10 and 6) The relationship between values for weight of the fully grown mass and the number of its constituent hairs was close in males and females of both ethnic groups, for example, the coefficients of correlation were +0.924

($P < 0.001$) in Japanese males and +0.941 ($P < 0.001$) in Japanese females.

c. Mean length and breadth of the 3 longest hairs in shavings of the full grown mass of hairs in the right axilla (Figs. 12–15. These hairs were longer ($P < 0.001$) in Caucasian than in Japanese, as tested

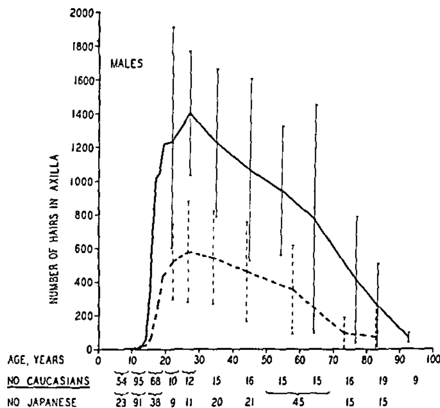


FIG 10 Mean number of hairs in right axilla of males

between 20 and 89 years of age These differences were apparent at all ages (except for females in the second and third decade of life)

As tested between 20 and 89 years of age the hairs were also significantly ($P < 0.001$) broader in Caucasian than in Japanese females, but the breadth was greater ($P < 0.001$) in Japanese than in Caucasian males

d Calculated mean weight of individual axillary hairs (Figs 16 and 17). The calculated mean weight per axillary hair was estimated for each subject by dividing the weight of the fully grown mass by the

number of its constituent hairs. This calculation is influenced by the fact that the hairs at the periphery of the axilla are smaller, appear later, and disappear earlier than the larger hairs in the center of the axilla. Obviously, changes with age in values for the calculated mean weight per hair are in some part a reflection of differences in the composition of the population of hairs.

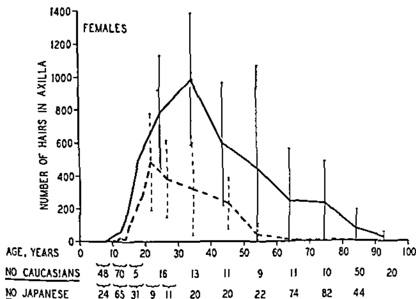


Fig 11 Mean number of hairs in right axilla of females Because of cosmetic practices, fully grown masses of hairs were not made available by many of the Caucasian females Probably as a result of the limited number of subjects none of the Caucasian females in the fourth decade had counts as low as those in some subjects at all other ages Therefore, the occurrence of peak values in the fourth decade (instead of in the third decade as observed in other types of measurements with larger groups of females) is considered to be spurious

In males between 20 and 59 years of age the calculated mean weight for individual hairs was significantly lower ($P = 0.01$) in Caucasians than in Japanese In females (Fig 17) the curve of means was not markedly dissimilar for the two ethnic groups until the age of the menopause, after which many Japanese women lost all axillary hair, a status which was recorded as a zero value

It is concluded that the greater weight of axillary hair shavings in Caucasians than in Japanese is due more to a markedly larger number of coarse hairs than to smaller dimensions and weight of the individual

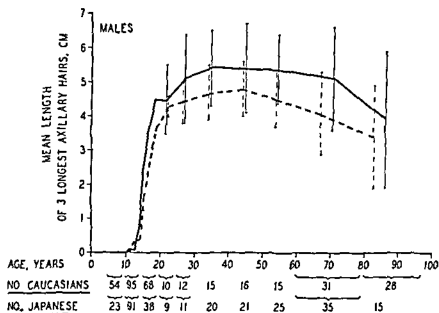


FIG 12 Mean length of the 3 longest hairs in shavings of the fully grown mass of hairs in the right axilla of males

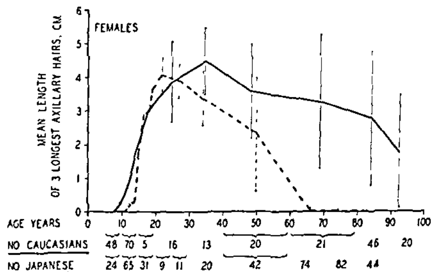


FIG 13 Mean length of the 3 longest hairs in shavings of the fully grown mass of hairs in the right axilla of females The low curve of means for old Japanese women is due to the fact that many Japanese females lost all axillary hairs after the menopause, a status recorded as zero

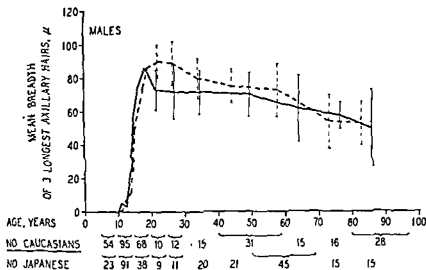


FIG 14. Mean breadth of 3 longest hairs in shavings of the fully grown mass of hairs in the right axilla of males. Values for subjects with no hairs were recorded as zero

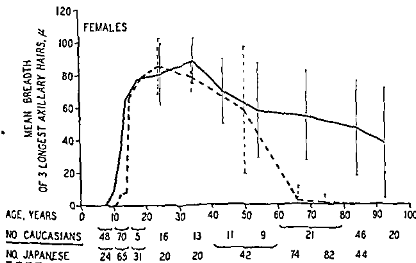


FIG 15 Mean breadth of 3 longest hairs in shavings of the fully grown mass of hairs in the right axilla of females Values for subjects with no hairs were recorded as zero.

coarse hairs. A similar conclusion was drawn from studies of the weight and breadth of beard hairs in males of these two ethnic groups.

e. Percentage of gray hairs. In Caucasian, as compared with Japanese males (Fig 18), graying tended to occur earlier and to a greater extent ($P = 0.02$, as tested in subjects 40 and 89 years of age). The extent of the disparity between the ethnic groups is shown much more clearly in median than in mean values since the latter are dis-

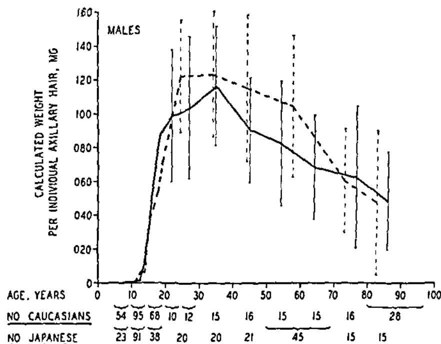


FIG 16 Calculated mean weight per individual hair of the right axilla of males.

torted by data for 3 Japanese men who were extraordinarily gray, even grayer than any of the Caucasian men of comparable age

In Caucasian females (Fig. 19) a small percentage of the hairs were gray in the fifth and sixth decades of life, whereas all hairs were black in Japanese females In women, 60 or more years of age, the comparison of ethnic groups was limited by the absence of axillary hairs in 196 of the 200 Japanese subjects Even in the 4 older Japanese women who did have axillary hairs the number of hairs was so small that the representativeness of the data for grayness is questionable With these reservations in mind it may be stated that mean and median values for

the incidence of grayness tended to be higher in Caucasian than in Japanese females.

Among Caucasians sex differences in grayness were apparent after the sixth decade. The females were significantly grayer ($P = 0.01$) than males, as tested in 89 subjects 60 to 89 years of age (compare Figs 18 and 19). In the Japanese the studies of sex differences in the graying of axillary hairs was handicapped by the great reduction in

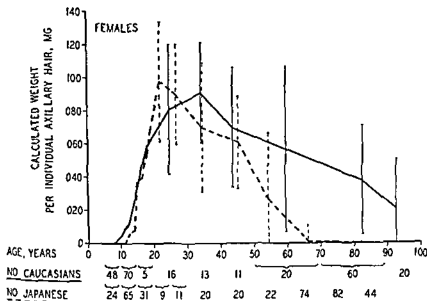


FIG 17. *Calculated mean weight per individual hair of the right axilla of females* The curve of means for the older Japanese females is low because many of these women lost all axillary hairs after the menopause and their values were recorded as zero

number of hairs that occurred in females after the menopause as well as by the fact that grayness was considerably less frequent in axillary than in beard hairs (compare Figs 5 and 18).

f Other measurements Studies of axillary hairs by several techniques, including those for the area covered by coarse hairs and the number of kinks *per unit length of hair, are incomplete but the data* obtained thus far confirm the findings with other techniques to the effect that the sequence of retrogressive changes with advancing age was in some regards the reverse of the order observed upon sexual maturation The forms of atrophy associated with aging occurred more

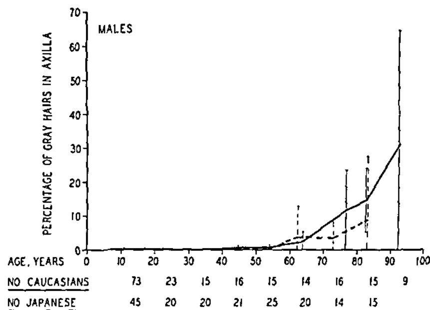


Fig 18 Mean percentage of gray hairs in shavings of the fully grown mass of hairs in the right axilla of males

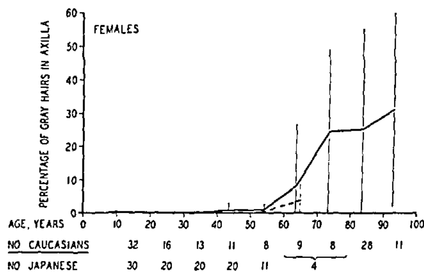


Fig 19 Mean percentage of gray hairs in shavings of the fully grown mass of hairs in the right axilla of females Axillary hairs were absent in most Japanese women after the menopause and no values for gray hairs were recorded in these subjects

extensively and with greater rapidity after removal of the testes and are considered in the section pertaining to eunuchs.

C. *Acquisition of Common Baldness of the Scalp and the Development of Coarse Hairs on the External Ears*

Brief reference may be made to differences between Caucasian and Mongolian populations in other matters involving pilosebaceous apparatus With ageing, *one or both of the pilary states of common baldness* and auricular hirsutism appear in some men and progress to various degrees (Hamilton, 1946, 1951b), whereas in females, baldness is uncommon (Hamilton, 1951b) and coarse auricular hairs are never present under normal conditions (Hamilton, 1946).

In males advanced degrees of common baldness are more frequent and develop at an earlier age in Caucasian than in Mongolian populations. Coarse hairs on the external ears are more numerous and larger in Caucasian than in Mongolian males (Hamilton, 1946).

A somewhat analogous difference involving the pilosebaceous apparatus is the higher incidence of advanced stages of the pathologic state, acne. *Severe acne is more frequent in both male and female* Caucasians than in Japanese of comparable sex and age. Dietary differences cannot be excluded as a complicating factor but are in themselves not a complete explanation, since the disparity in values between Caucasians and Japanese was greater in the female than in the male sex, as was also the case in growth of axillary and beard hairs

In brief, extensive development of these three male-selecting traits is more common, and occurs at an earlier age in Caucasians than in Japanese

D. *Studies of Caucasian Eunuchs*

The eunuchs under investigation were inmates of a home for the feeble-minded In many feeble-minded subjects, quite apart from those who have also undergone gonadectomy, values for many traits used as endocrine indicators tend to be significantly lower than in males of normal mentality. This is illustrated in Fig 20 by data for weight of beard. After acknowledgment of this phenomenon it may be noted that, even in the feeble-minded, castration resulted in a further significant decrease in weight of beard grown per day, in area covered by coarse hairs, and in number of such hairs per square centimeter of cheek

1. *Influence of Age of the Subject at Castration*

The beard grew but little in subjects operated before 16 years of age and tended to be progressively heavier the longer the interval be-

tween sexual maturation and the age at castration (Fig. 21). When orchiectomy was delayed until 21 or more years of age, the beard weights were considerably higher than in subjects castrated by 20 years of age (Fig. 22), values remained higher throughout life in the late than in the early operated group (Fig. 20). The heaviest weights of beard were observed in men who had not been castrated until 30 or more years old (Fig. 22).

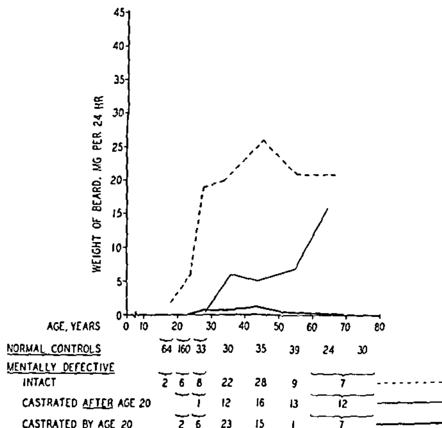


Fig. 20. Mean weight of beard grown per day by eunuchs. Note the particularly poor beard growth in males castrated at or before 20 years of age, i.e., prior to extensive development of the beard. As a result of orchiectomy, growth was reduced even in the feeble-minded, a group with subnormal values before castration as shown by data for intact feeble-minded males vs. those for males of normal mentality. The last point on the curve of averages for eunuchs castrated after 20 years of age is exceptionally high because many of the subjects in this age group had been castrated at a late age when they had developed extensive beards.

Prolongation of the time between sexual maturation and castration also resulted in sustained increments for breadth of coarse beard hairs (Figs. 22 and 23). Deferment of castration after 29 years of age, however, produced little further augmentation of breadth beyond that obtained with postponement of operation until this age (Fig 22). Thus the heavier weight of beard observed when the operation had been delayed after the third decade is attributable only in small part to

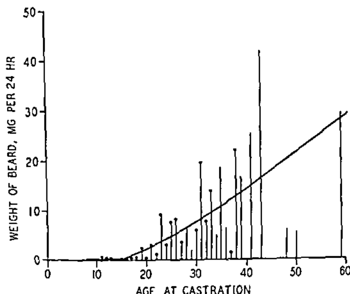


Fig 21. Increase in weight of beard grown per day with prolongation of the interval between sexual maturation and castration The curve of means, smoothed through the plotted points by the method of least squares, is based upon data for 108 eunuchs Vertical lines which terminate in knobs represent the average for two or more subjects of the same age

greater breadth of the coarse hairs (Fig 22), presumably an increase in the number of coarse hairs is the chief factor

The increase in breadth of hairs upon extension of the interval between maturation and castration (Fig 23) was due to greater breadth not only of the cortex and cuticle but also of the medulla In absolute values the increase in diameter was about the same in the medulla as in the rest of the hair.

2. *Influence of the Interval Since Castration*

Zero-order correlations of data for 31 men who had been gonadectomized by 20 years of age suggested ($P = 0.04$) that the breadth

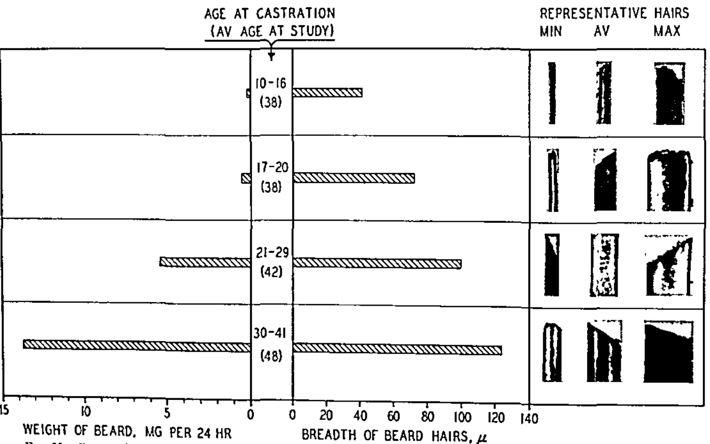


Fig. 22 Data and representative hairs for 41 eunuchs, arranged in 4 age groups, indicating that castration during adolescence suppressed the ordinary increments with age in weight of beard grown per day more than it inhibited the increase in breadth of the coarse hairs

of coarse beard hairs increased with ageing of these men while they were in a castrate state. The statistical procedure of partial correlations also indicated that the breadth of hairs increased ($P = 0.006$) during the castrate state. However, the weight of the beard grown per day did not increase significantly with ageing of these eunuchs while they were in a castrate state.

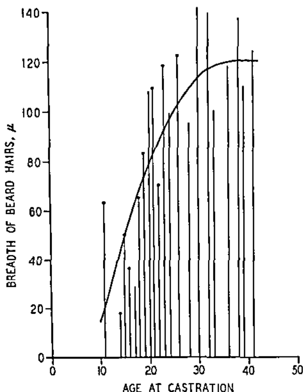


FIG 23 Increase in breadth of coarse beard hairs with prolongation of the interval between sexual maturation and castration Data and smoothed curve of averages are for 41 eunuchs Consult legend of Fig 21 for details

It would appear that beard hairs have an inherent tendency to become somewhat coarse with age even in the absence of gonadal secretions and in spite of a known decline in the 17-ketosteroids derived from extragonadal sources Urinary titers of 17-ketosteroids were subnormal in the young eunuchs and further reduced in older eunuchs Moreover, values for urinary 17-hydroxycorticosteroids were found to be similar in castrate and intact men Thus, assessment of adrenocortical function

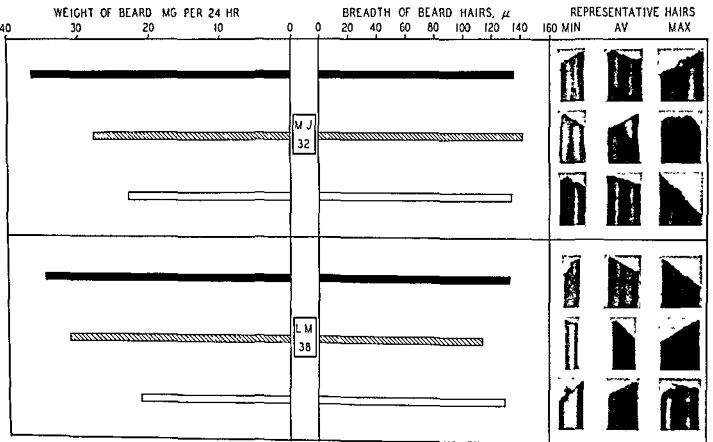


FIG 24, Part 1 (See following pages for Parts 2 and 3)

by both methods of assay provides strong evidence that steroids derived from extragonadal sources did not exceed normal values and therefore were not responsible for the increasing coarseness of beard hairs with advancing age.

The increase with age in thickness of facial hairs in eunuchs is in keeping with a similar phenomenon observed in young and middle-aged women (Trotter, 1942). We have previously demonstrated a statistically significant tendency to an increase with age in the thickness of another integumental appendage, the nail (Hamilton *et al*, 1955) This is not a universal property of integumental appendages, however, since there is a decrease with age in thickness of hairs of the scalp (Wynkoop, 1929).

3 Area of Face and Neck with Coarse Beard Hairs and the Number of Coarse Hairs Per Square Centimeter of a Standardized Region of Cheek

Values were markedly low in eunuchs, particularly in those orchiectomized by 20 years of age.

After castration of adult men, retention of coarse beard hairs tends to be greatest in regions where hair develops initially (Danforth, 1925) upon sexual maturation These sites are the upper lip, especially its lateral portions, the center of the chin, and the skin immediately anterior to the ears

4 Observations of the Same Eunuchs Before and After Castration (Fig. 24)

It was possible to study a few males before and after castration Since such subjects serve as their own controls, variation is not introduced by comparison of data for different groups of individuals

These studies provide further evidence that, after castration, some of the late sequelae of sexual maturation tend to regress earlier than phenomena that appear soon after puberty. The weight of beard grown per day, maximal values for which are not attained until the fourth decade of life (Fig 1), decreases more readily than the mean breadth of coarse hairs (Fig 24), for which values are high by the third decade of life (Fig. 4) Some of the late sequelae of sexual maturation disappear entirely after castration, such as the growth of coarse hairs in regions at a distance from the primary centers of hair growth

Orchiectomy exerts somewhat parallel effects upon growth of axillary hairs. It is known (Crampton, 1908, Danforth, 1925, Greulich *et al*, 1942) that upon sexual maturation, hairs develop first in the central portions of the axilla and later at the periphery, these hairs also

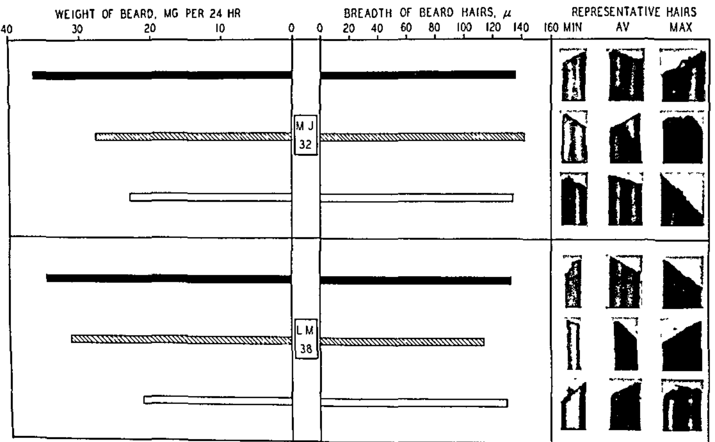


FIG 24, Part 1 (See following pages for Parts 2 and 3)

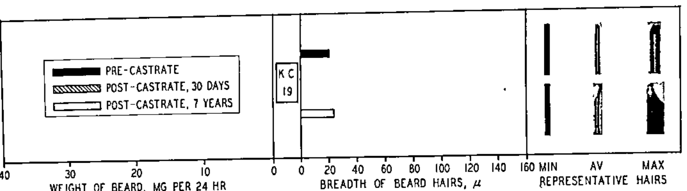


FIG 24 (Part 3)

FIG 24 Comparative studies before and after castration in 5 males and photographs of representative beard hairs. Note reduction in weight but not in breadth of hairs

acquire pigmentation before they become kinky. Following gonadectomy in adults the size of the hairy area is reduced centripetally, and the hairs that remain tend to lose their kinkiness while they retain some pigmentation.

III. Discussion

A. Interpretation of the Observation that Graying of the Beard and Axillary Hair Occurs Earlier in Caucasian than in Japanese Males

The graying of hairs is commonly regarded as an index of ageing. What is responsible for earlier graying of the beard and axillary hair in Caucasian than in Japanese males? Possible factors include differences in the size of hairs, methodologic difficulties in distinguishing which hairs are gray; the effect of the initial degree of pigmentation upon rate of graying; differences of a genetic nature, and nongenetic influences upon rates of ageing such as obesity

The diameter of hairs, which is commonly claimed (Terada, 1956) to bear a relationship to graying, is not responsible for the differences observed between ethnic groups in the present study As tested in men 30 or more years of age, the mean breadth of hairs was greater in Caucasians than in Japanese for beard, whereas the opposite was the case for axillary hair.

The coarse hairs were uniformly black in the Japanese, whereas in Caucasians they were of various hues and usually lighter in color Difficulties in distinguishing the hairs which are gray do not seem to be a major source of error, however, since the omission of data for Caucasians with blond hairs did not alter the incidence of graying observed in the entire group of subjects.

Subjective errors in determining which hairs were gray do not appear to have been consequential, perhaps partly because decisions were made under standardized lighting and by the same two persons.

The reliability of the evaluations was tested in triplicate studies of the same shavings of beard from 13 males with a range of 19 to 86 years Periods of several months intervened between each of the three examinations The triplicate values were in good agreement, with a mean coefficient variation of 10%

The earlier graying of hair in Caucasian than in Japanese men might be interpreted as another facet of the phenomenon evident in many of the data reported in this communication, to wit, a tendency to greater development in Caucasians than in Japanese of many secondary sex differences. Terada (1956) has shown that in Japanese subjects graying of the hairs in many bodily sites occurs earlier in males than in females.

We have observed considerable similarity between identical twins in graying of the hair. Familial tendencies to graying are common knowledge. It seems wisest to consider, tentatively, that genetic factors are responsible for the differences in graying between the ethnic groups, but the present data do not eliminate nongenetic influences such as obesity that might affect the rates of ageing.

B Factors Regulating Growth of Hairs in Regions Under Study

1 Beard

That the beard is a male secondary sex character is attested by the following facts Under normal conditions it achieves full development only in males. It fails to grow in eunuchs castrated prior to puberty, and undergoes regression after orchiectomy of mature males. Growth of whiskers is stimulated by androgenic treatment in eunuchs and old men (Chieffi, 1949).

Despite dependence of the beard upon androgenic stimulation for growth and maintenance, the present observations of ethnic groups and twins show that genetic factors exert a major degree of control over the extent of beard growth. An extensive beard owes far more to genetic predisposition, abetted by age (and possibly by prolongation of the period of androgenic stimulation) than it does to quantities of androgens in circulation that are in excess of those in the mature man with a sparse beard. This almost triggerlike action of physiologic quantities of androgens is similar to the situation demonstrated in earlier studies of common baldness and acne (Hamilton, 1941, 1942, 1951b), namely, the promotion by androgens of phenomena the very occurrence and extent of which are controlled by genetic, ageing, and other factors [3]

The interrelations of endocrine, hereditary, and ageing factors observed in studies of the beard seem relevant to coarsening of hairs in many other regions such as eyebrow, nasal vestibule, external ear, in fact in most of the body with the exception of the scalp, axilla, and pubis.

2 Axillary Hair

Coarse axillary hairs are secondary sex characters since they appear only upon sexual maturation, fail to grow in men castrated prior to puberty, and are not maintained either in number or in a fully developed state with advancing age or after castration of adults (Hamilton, 1951a)

[3] There are dissimilarities as well as similarities in the factors which regulate the pilary states of beardedness and baldness, as shown by their differential incidence in males and by differences in the permanence of the conditions once established.

In maturing boys, axillary hairs appear first as a central tuft that comes later to extend centrifugally. The hairs begin as fine silky structures, then acquire pigment, and finally become kinky (Crampton, 1908); these steplike stages are associated with increments in titers of urinary androgens (Greulich *et al.*, 1942). This sequence of changes in axillary hair at puberty tends to be reversed with ageing or castration. The hairs fail to be replaced, especially around the periphery of the tuft, and many of the remaining hairs decrease in kinkiness, thickness, length, and somewhat in pigmentation. Retrogressive changes after castration occur more rapidly than those which accompany ageing, the atrophy is also more extensive in eunuchs than in intact subjects until the latter reach very old age.

The atrophy of axillary hairs with ageing may be presumed to relate in part to a body-wide loss of cells (O'Leary, 1952) and in part to the known slackening of gonadal secretions (Hamburger, 1948, Hamburger *et al*, 1945, Hamilton *et al*, 1948, 1954). The mean age curve (Fig 6) for weight of axillary hair in Caucasian males corresponds to the mean age curve for titers of urinary androgens in these men (Hamburger *et al*, 1945, Hamilton *et al*, 1954) But in individual subjects the relationship of axillary hair growth to titers of urinary androgens and ketosteroids is poor, perhaps because of genetic differences in the extent to which axillary hairs grow in response to stimulation.

3 *Usefulness of Quantitative Measurements of Beard and Axillary Hair in Studies of Endocrine, Genetic, and Ageing Processes*

Measurements of beard and axillary hairs can serve as assay procedures Growth of the beard is chiefly an indicator of maleness, whereas axillary hair is stimulated by estrogens as well as androgens Under ordinary circumstances, growth of hair in both of these regions is almost completely dependent upon testicular and ovarian secretions

Growth of the beard and axillary hairs, like growth of the comb in fowl, sums up responsiveness of the organism as well as the stimulation provided In our studies of other secondary sex characters and organs, and of sex-selective pathologic traits, the responsiveness both of target organs and the organism was found to be of great importance (Hamilton, 1941, 1942), a similar phenomenon has been reported in animals (Emmens, 1939, Gardner, 1955)

Beard and axillary hair, the growth of which is a summated response to several factors, provide useful tools in genetic and gerontologic studies Component factors can be varied separately, for example, the influence of various degrees of genetic predisposition can be analyzed by studies of twins and other siblings Manipulation of certain environ-

mental factors is also possible For instance, males of Japanese ancestry who lived in New York City and ate American diets had values for beard and axillary hair similar to those in men of comparable age who lived in Tokyo and adhered to Japanese modes of diet (Hamilton *et al.*, 1958). It can be concluded that geographic, climatic, and dietary factors are of no major etiologic importance in the lesser growth of beard and axillary hair in Japanese than in Caucasians.

C. Spectrum Extending from Tendencies to Highly-Developed Secondary Sex Characters to Certain Pathologic States, Rapid Ageing, and High Mortality Rates

Values for the beard, and those for any endocrine indicator studied thus far, do not assess all aspects of maleness and do not correlate closely with data for one another. Indeed, the responsiveness of different endocrine indicators may alter in opposite directions upon ageing The beard is slow to develop but well maintained in old men despite a demonstrated decline in testicular secretions In contrast, values for axillary hair, or for acid phosphatase in prostatic exprimate (Kirk, 1952), attain a peak more quickly after sexual maturation and undergo retrogression after young adulthood, these changes conform more closely than in the case of the beard, first with the flow and then with the ebb of gonadal secretions

The respective roles played by various factors in the regulation of beard growth, and of other endocrine indicators, suggest principles which deserve cautious evaluation of their applicability to pathologic states involving pilosebaceous and other structures It must be fully appreciated, however, that many secondary sex characters, as well as sex-selective pathologic states, exhibit considerable specificity in responsiveness, age at development, and manner of regulation

With the above comments in mind it is of interest (a) that prepubertally castrated males develop neither secondary sex characters, such as beard and axillary hair, nor the pathologic states of common baldness and acne to which males are particularly susceptible, (b) that in intact males the Japanese, as compared with Caucasians, tend to lesser growth of these same secondary sex characters, to a lower incidence of severe forms of common baldness and acne; to slower ageing as indicated by retained pigmentation of hairs, and to lower mortality rates (U.S Bureau of Census, 1955), and (c) that also in females the Japanese, as contrasted with Caucasians, exhibit lesser growth of facial and axillary hair, fewer instances of severe acne, and lower mortality rates (U.S Bureau of Census, 1955).

If the modes of regulation of secondary sex characters are partially

analogous to those of the highly important sex-selective pathologic states, the specificities exhibited by these characters and their component parts deserve study with regard not only to their nature but also to their degree of plasticity, changes with age, and responsiveness to environmental factors. As an illustration, beard growth in intact subjects, once established, persists as do the male form of genitalia and the pattern of baldness of the scalp. The development of male genitalia is induced prenatally by testicular secretions in mammals (Burns, 1955, Jost, 1953) and requires a plasticity of embryonic structures that disappears in later life Baldness of the scalp represents the other extreme, a condition more readily elicitable with advancing postnatal age. It may be expected that various combinations of factors will be operative in different entities, and to varying degrees at different ages and that these factors will include environmental influences such as nutrition and stress.

IV. Summary

Techniques have been formulated to provide repeatable quantitative measurements of hairs and their rate of growth in certain regions of the body With these methods data have been obtained for each sex throughout the lifespan in Caucasian and Japanese populations. The standards constructed from these values can serve to assess some aspects of physiologic age, to study endocrine status, and to gain further understanding of the nature and interdependence of endocrine, ageing, genetic, environmental, and other regulatory factors

Beard growth, in Caucasian as compared with Japanese males, was found to be considerably greater and peak values were attained earlier in life This was shown by measurements of weight of hair grown per day and was associated with higher values in Caucasians for the area of skin with coarse hairs and for the number of such hairs per square centimeter of a standardized region of the cheek. The breadth of individual coarse hairs was similar in the two ethnic groups. In parallel with the lesser growth of beard in Japanese than in Caucasian males, no instance of facial hirsutism was found in a large series of Japanese females whereas a high incidence is reported for Caucasian women

Growth of axillary hair was also more pronounced in Caucasians than in Japanese of comparable age, as tested separately for each sex. The disparity in values between the two ethnic groups was even greater in females than in males.

These data for beard and axillary hair are in keeping with a greater tendency on the part of males of Caucasian than of Mongolian populations, to certain other changes in integumental appendages, such as

the development of coarse hairs on the external ears, the higher incidence of extensive baldness of the scalp, and the greater frequency of severe acne

Graying of the beard and of axillary hair occurred earlier in Caucasian than in Japanese males.

Studies of eunuchs showed that secondary sex characters and certain sex-differing pathologic states fail to develop in men who do not mature sexually. These traits and states, once fully developed, differ in the degree to which their maintenance depends upon continued secretions of the testes In decreasing order of dependence are axillary hair, beard, and common baldness.

Although the traits under consideration are ordinarily dependent upon gonadal secretions for development, and in some instances for maintenance, the requisite endocrine stimulation tends to act somewhat in a triggerlike fashion. The extent to which these states develop, and even the occurrence of certain sex-selective pathologic states, is regulated chiefly by inheritance and ageing. Studies of twins and members of large families, supplemented by comparisons of Caucasians and Japanese (including Japanese living in Tokyo vs New York), delineate and emphasize the large measure of control exerted by genetic factors

Endocrine indicators of the quantitative type employed in these studies are thus to be regarded as somewhat analogous to the comb in fowl, reflecting not only the nature and type of the existent endocrine stimulation but also the vitally important responsiveness both of target organs and the total organism.

The interrelations of endocrine, hereditary, and ageing factors observed in studies of the beard seem relevant to growth of hairs in many other regions such as eyebrow, nasal vestibule, external ear, and in fact much of the body with the exception of scalp, axilla, and pubis.

The mean age-curves for axillary hair conform more closely than those for beard to the waxing and waning of gonadal secretions, as judged by titers of urinary

Secondary sex character male-selecting pathologic states severe forms of acne There are suggestions that this spectrum may extend to some of the more lethal male-selecting pathologic states Seemingly, there is a spectrum in which tendencies to highly developed secondary sex characters merge with tendencies to sex-selective pathologic states, rapid ageing, high mortality rates, and a short duration of life. Throughout this spectrum these tendencies are greater in males than in females, this is so in both Caucasian and Japanese populations. The tendencies are also greater in Caucasians than in Japanese, in comparisons either

between males or between females. Furthermore, intact males outstrip eunuchs in the degree of development of secondary sex characters and in the incidence of certain pathologic states.

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CHAPTER 17

Physical Factors Which Influence the Growth of Hair[1]

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I. INTRODUCTION

In hair follicles, there are certain conditions and processes which are subject to certain influences and others which are relatively immune to them. Hair growth is cyclic in terms of each follicle Presumably, hair production could be increased by the (1) initiation of a new anagen in a telogen follicle, the (2) delay or complete prevention of telogen, the (3) transformation of a follicle into one having longer anagen and shorter telogen phases, the (4) production of new follicles or multiple follicles, i e increase in the number of hairs per unit area, and the (5) increase in the anagen growth rate of a follicle

Taking the above categories in reverse order, number (5), the increase in growth rate, cannot be achieved to any pronounced degree. The anagen follicle seems to be producing hair at a near-maximal rate. Warmer temperatures may increase the rate slightly (Trotter, 1923), but this is doubtful If there is any diurnal variation in rate (not found in the mouse and probably not in man), the lower rate might be abolished Semi-starvation only slightly reduces the rate of growth of an already growing follicle in the mouse (Loewenthal, 1956) It is doubtful if any agent can increase sensibly the already extreme rate of mitotic activity in a follicle.

In the case of number (4), neogenesis of follicles in a sparsely haired area can possibly restore such an area to a normal population of follicles (Billingham and Russell, 1956) but the production of more than a normal population is not yet possible Multiple follicles, by some induction of twinning, might be possible eventually (cf Wilcox, 1950, for chinchilla, and Lyne, 1957, for sheep)

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In number (3), the transformation of a follicle into one having a longer anagen relative to its telogen phase would be of considerable practical and cosmetic value. In hereditary pattern baldness in the scalp of man follicles change from a type with a long anagen to one with a very short anagen. This change can be prevented by castration (Hamilton, 1942) but the reverse change has not been achieved. When more is known about the change in follicle type, the accumulation of inhibitors (Chase, 1955), or the access to stimulators of anagen, it may be possible to change the follicle back to its long anagen behavior.

In number (2), delay or complete prevention of telogen is not yet experimentally feasible. Perhaps the normal accumulation of an inhibitor can be prevented. Incidentally, in pattern baldness in man, the transformation to the new type of follicle, seems to occur at telogen, the hair lost being a club hair If a follicle could be prevented from reaching telogen the transformation might be prevented. Follicles in sheep have infrequent resting phases. Angora hairs in rabbits have a very short telogen, a stage which may even be bypassed entirely by clipping (Crary and Sawin, 1953). The prevention of telogen, whether it be by preventing the accumulation of growth inhibitors or by the maintenance of unknown stimulators may be practicable when more is known of the dynamics of the cycle of the follicle.

II. Factors Influencing Hair Growth

At present the only effective controls of hair growth are related to the first category, the initiation of a new anagen in a telogen follicle. Several methods are applicable at this point. Chemical irritants are effective (Rauch, 1952), as are also methylcholanthrene-in-benzene (Chase and Montagna, 1951; Chase, 1954), and probably anything that causes extensive epidermal hyperplasia. If about 1500 r of x-ray are delivered to the skin of the mouse with resting follicles new hairs grow from these follicles; lower doses are ineffective (Chase, 1958) This phenomenon also occurs in the rabbit (Jolles and Greening, 1957).

equally effective is the situation wherein the hair breaks off just above the club. In this case, however, the club, although remaining for a time, is loosened and falls out with the next growth of hair. In the mouse a club from which the hair has not been plucked can remain embedded even after several generations of spontaneous hair cycles have occurred. The similarity between experimental sloughing of epidermal corneum (Pinkus, 1952) and plucking of club hairs in terms of induced mitotic activity is striking and may be explained by loss of the normal inhibitors in the homeostatic systems (Chase, 1955, 1958). There is evidence that vigorous grooming, i e, pulling the hairs but just short of dislodging or breaking them, is effective sometimes in initiating anagen in the mouse. Vigorous massaging of the mouse skin, sufficient to produce erythema, does not initiate growth in the resting follicles.

Factors influencing hair loss must also be considered in relation to the hair cycle. The falling out of a growing hair is a different process from the falling out of a resting club hair. In the former case there will be an effect on the rapidly dividing cells of the matrix of the bulb In the latter, there will be an effect on the mitotically inactive cells of the capsule and anchoring club. A single painting of 0 6% methylcholanthrene-in-benzene, for instance, does not affect the growing hair follicle but causes depilation of resting hair by damaging the capsule (Chase and Montagna, 1951) This "damage" results in mitotic activity of the external sheath and capsule and consequently a new hair growth. Freezing (Taylor, 1949), severe heat, acid, in fact probably any severe treatment which damages the matrix of a growing follicle, causes the hair to stop growing, and with no club formed, depilation ensues Comparable treatments to resting follicles do not cause depilation unless the treatments also cause severe erosion of the skin, or mitotic activity to occur in the capsule and external sheath.

Although cutting or shaving, without pulling, has no effect on the hair cycle, dekeratinizing depilatory agents, such as barium sulfide, do have an effect. There is no effect on a growing follicle except the loss of the keratinized hair, but on a resting follicle it has the same effect as plucking.

Ionizing radiations well illustrate the difference between the epilation responses of growing and of resting follicles. An x-ray dose of 400 r causes temporary epilation at 4 days in growing follicles of mice (Chase, 1949, Argyris and Chase, 1956) through a disorganization of the vital structure of the bulb (Fig 1) A dose of about 1500 r is necessary to epilate a resting follicle 13–21 days after treatment (Argyris, 1954). Some follicles become permanently unable to produce a hair at doses of about 2000 r and higher (Chase, 1949, Geary 1952),

but *some of them are still competent, although greatly delayed, even* after 5000 r. Damage to early anagen, or "embryonic," follicles is particularly devastating, and although permanent epilation is not always the consequence, the time for restoration of a competent follicle is decidedly long. Strauss *et al.* (1954) have succeeded in protecting the

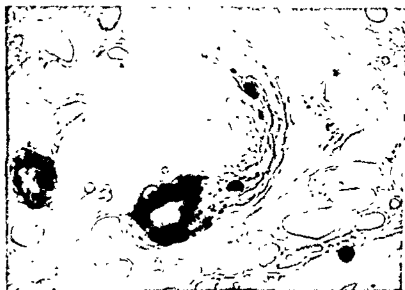


FIG. 1. Unstained section. This growing follicle received 1000 r 4 days previously. Note the disorganization of the bulb, the descent of melanocytes and the abortive keratinized hair produced after irradiation. Magnification × 300.

skin of mice against x-rays by making it anoxic with a surgical clamp and with injections of epinephrine. For studies on the effects of x-irradiation on human hair follicles, see Halberstaedter (1929) and Montagna and Chase (1956).

III. SUMMARY

In conclusion, the phase of the hair cycle at the time of the application of any physical factor is very important. Only one of the five possible ways of increasing hair growth is at present practical, i.e., the initiation of a new anagen from a telogen follicle. With more information on the controlling mechanisms of the hair cycle, however, it may be possible to delay or even prevent the onset of catagen-telogen and thus produce an ever-growing or at least angora type hair.

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CHAPTER 18

Response of Hair Roots to Chemical and Physical Influence

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I. Introduction

Certain chemical agents, ionizing radiation, and particular illnesses cause the loss of scalp hair. The type and extent of damage that occur in the hair root in each instance are not known The following account describes some of the morphological changes that have been observed. Certain types of hair loss may be identified on the basis of these changes.

II. Morphology of Normal Hair Roots of Scalp (Van Scott *et al*, 1957)

The structural pattern of hair roots can be seen well in hairs that are plucked from the scalp, immersed in water, and viewed microscopically by transmitted light. Hairs in anagen, catagen, or telogen phases of growth can be easily identified. The root of an *anagen* hair is characterized by the typical structure of its bulb, and the presence of the dark keratogenous zone immediately distal to the bulb (Figs 1, 2). Internal and external root sheaths may either be present and intact, partially present, or absent. A *telogen* hair has no keratogenous zone. Its proximal tip is club-shaped and is keratinized. Neither internal nor external root sheaths are present, the hair club being surrounded by the epithelial sac (Fig 2). A *catagen* hair also has a club-shaped, keratinized tip, but is distinguished from a telogen hair by the presence of both an internal and an external root sheath (Fig 2)

Normally, the proportion of anagen hairs in randomly plucked hairs

[1] National Institutes of Health, Public Health Service, U S Department of Health, Education and Welfare

of the scalp is 85–90% of the total. The range of this percentage may be considerably varied and may be as low as 65% in some individuals and as high as 95% in others. Catagen hairs are infrequently found in the scalp, normally accounting for less than 1% of the total. The likelihood of extracting a hair in any given phase of the growth cycle



FIG. 1. Roots of manually extracted *anagen* hairs of the scalp, viewed microscopically by transmitted light. A. Root with sheaths intact. A portion of the connective tissue hair papilla remains attached to the matrix portion of the bulb, a circumstance only infrequently found in plucked hairs. B. Root of a hair with only its internal sheath intact at the bulb. C. Root of hair with its internal and external sheaths torn at the level of the keratogenous zone.

is proportionate to the duration of the cycle concerned. The time required for a hair to pass through catagen, the transitional phase between anagen and telogen, is short in comparison to either anagen or telogen. Hence, most hairs of the scalp are found to be anagen and telogen in type.

III. Hair Roots Following Methotrexate (Amethopterin)

Changes that occur in scalp hair roots have been studied in patients who have received this folic acid antagonist as therapy for neoplastic diseases (Van Scott *et al.*, 1957). The dose of the drug effecting changes in the hair root varies from individual to individual. The degree of

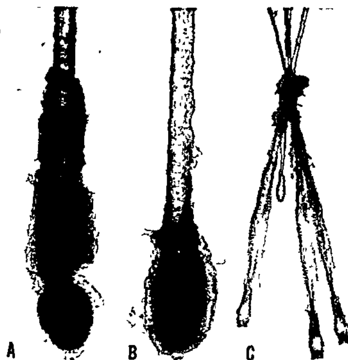


Fig. 2 Comparison of different stages of the growth cycle in roots of hairs extracted from the scalp A. Hair root in catagen (involutional) phase of the growth cycle. The external sheath and internal sheath are present The bulb, however, is keratinized and a keratogenous zone cannot be detected B Hair root in telogen phase of the growth cycle (club hair) The external sheath, internal sheath, and keratogenous zone are absent The keratinized bulb is surrounded by the epithelial sac C A group of four hairs that had emerged from the skin through a common follicular pore The three lower hair roots are anagen in type, the upper one is telogen in type

effect on the hair in a given individual is further dependent upon the route and schedule of administration of the drug

Four days following a single large intravenous dose of methotrexate a diminished diameter of the bulb may be detected in anagen hairs

extracted from the scalp. The degree to which the diameter is diminished *does not, however, foretell the severity of changes that may subsequently* appear in the hair shaft. Six days following the single dose, and thereafter, a constriction in the hair shaft is evidence of the previous insult to the hair bulb (Fig 3). When the drug is no longer administered the hair bulb abruptly recovers and resumes producing a hair

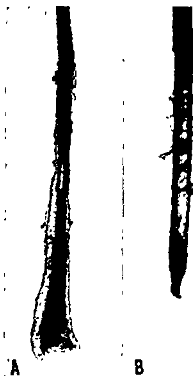


Fig 3 Effects of methotrexate (amethopterin) on anagen hair roots of the scalp A Constriction in the shaft of a hair following methotrexate B Hair shaft which has broken off at a site of severe constriction caused by methotrexate

shaft whose diameter is again normal The constriction subsequently moves *distally* as the hair continues to grow *In a given patient, the* degree of constriction is proportionate to the amount of drug received If the dose is sufficiently large the degree of constriction is severe, and the hair shaft may break at the zone of constriction when the hair is pulled or casually manipulated as in combing or brushing (Fig 3) *The rate of production of hair does not appear to be greatly disturbed,* so that a focal constriction in the hair shaft occurs following a single dose of the drug, whereas an extended zone of diminished diameter

appears in shafts of hair following more prolonged courses of the drug. In no case observed has the injury of the hair bulb been severe enough to interfere permanently with the growth of hair. Weeks to months following a single dose or short course of the drug, the time of administration of the drug may be reestablished by dividing the distance (in millimeters) between the site of constriction in the shaft and the hair bulb by 0 35 mm, the average normal daily rate of growth of scalp hair (Myers and Hamilton, 1951; Trotter, 1925).

Morphological changes do not occur in roots of telogen hair inasmuch as they are mitotically, and presumably metabolically, at rest.

IV. Hair Roots Following Ionizing Radiation

In contrast to the reversible effects of methotrexate on the roots of growing hairs of the scalp, the effect of ionizing radiation generally is progressive (Van Scott and Reinertson, 1957).

Roots of extracted hairs first show detectable alterations 4 days following irradiation of the scalp area in which they were growing. The initial change consists of thinning of the hair bulb (Fig 4). Melanin pigment, normally confined to the portion of the hair bulb that gives rise to the cortex of the hair, is dispersed and may be seen in the internal root sheath The entire hair bulb undergoes a progressive atrophy during the next few days. Approximately one week following radiation, the hair bulb is so disintegrated that it no longer remains attached to the extracted hairs At 2–3 weeks following radiation the root portion of extracted hairs are tapered, and appear to be entirely keratinized Neither the internal root sheath nor the external root sheath are extracted with the hair at this time

The progressive atrophy of the hair root described above occurs in almost all anagen hair roots that show any visible effects of radiation The roots of a comparatively few hairs partially recover from the effects of radiation and continue producing hair. The hair shaft produced, however, is left with a constricted zone similar to that following methotrexate. This type of defect in hairs following radiation was noted as early as 1906 by Williams

The response of most anagen hairs to radiation seems to be predominantly an all-or-none phenomenon. The exceptional hair that appears to recover may represent a partial response to radiation, this, however, is not correlated with the dose of radiation On the other hand, there is a correlation between the dose (at least up to 300 r) of radiation received by the hair roots and the percentage of *anagen* hairs showing morphologic changes (Van Scott and Reinertson, 1957). The percentage

of hairs so affected has been found to be directly proportionate to the dose of radiation, and also to the time interval following radiation.

No morphologic changes have been observed to occur in hair roots following irradiation of neighboring areas of skin or distant parts of the body.

Degeneration of hair roots of the albino rat following epilating doses of x-rays has been studied histologically by Geary (1952) who de-



FIG 4 Effects of x-rays on anagen hair roots of the scalp A Appearance of anagen hair root 4–6 days following radiation Beginning atrophy of the bulb is apparent Granules of melanin pigment are dispersed in the surrounding internal root sheath B Hair root 6–8 days following radiation Atrophy of the bulb is severe The keratogenous zone is visible C and D Hair roots 3 weeks following radiation, showing complete atrophy

tected changes in the matrix of the hair bulb as soon as one day following exposure Montagna and Chase (1956) have described the histological changes occurring in the scalp of man following epilating doses of x-rays These changes are comparable to those detectable in manually extracted hairs They further described an interesting phenomenon occurring in the connective tissue sheath surrounding the hair root, the vitreous membrane. Within the first few days following radiation the

membrane becomes wrinkled and markedly thickened and remains so for several weeks thereafter. Normal morphologic appearance of the membrane is regained 10 to 11 weeks later, at which time the entire follicle again appears normal and regrowth of hair occurs.

No structural changes are known to occur in telogen hairs following doses of x-rays that cause temporary epilation.

V. Hair Roots in Alopecia Areata

Changes occurring in scalp hair roots in this disease are included here for comparison with those resulting from methotrexate and ionizing radiation

The etiology of alopecia areata is unknown. Loss of scalp hair begins either abruptly or gradually, leaving circular areas of alopecia that may enlarge to involve the entire scalp. Hair loss may occur on other parts of the body, but is first apparent and most conspicuous on the scalp

Sabouraud (1929) has called attention to the fact that the hair follicles in this disease repeatedly attempt to produce hair Roots of hairs that spontaneously fall out are markedly diminished in diameter and their proximal ends are pointed These are similar to hairs 2–3 weeks following epilating doses of x-ray. The root portions of plucked hairs in alopecia areata are thinner than normal Their attenuation may be either uniform or markedly irregular. The bulbs of such hairs may be small, but otherwise appear to be normal, or they may show varying degrees of atrophy and distortion. These changes resemble those produced by methotrexate In the latter instance, the hair bulb abruptly recovers and resumes producing a normal hair when administration of methotrexate is discontinued The hair bulb in alopecia areata appears to be under adverse influences of varying severity, over an extended period of time. When remission of the disease occurs, the bulb apparently recovers completely, as in the case following methotrexate

Histological observations (Van Scott, in press) show that most hair roots in alopecia areata are in an anagen phase of the growth cycle. The hair roots produce a morphologically normal internal root sheath, but the hair shaft produced is diminutive, and incompletely keratinized Such roots in alopecia areata are comparable to those of a particular stage of *early* anagen seen normally. This stage has been identified and described in the skin of the mouse by Chase *et al* (1951) who termed it anagen IV The stage is normally transient, and is quickly followed by the production of a fully keratinized hair of normal diameter In alopecia areata the hair bulb appears to be restrained from proceeding beyond the comparable stage of anagen IV and fails to produce morphologically normal hair

VI. Hair Roots Following Illness

This type of hair loss has been particularly associated with high fevers accompanying infectious diseases Abnormalities of the hair that Pinkus (1917) described in postinfectious hair loss include thinning of the hair shaft and hair root, and loss of pigment in the hair. The author has not observed these changes in cases where hair loss has occurred in the absence of infection. Where loss of hair has occurred in cancer patients without fever, in post partum patients who have not had fever, and in patients who have had high fevers without apparent infection, the abnormality has consisted only of an increase in the proportion of telogen hairs.

The kind and extent of changes that occur in hair following illness seem to depend upon the disease condition involved, and its severity.

VII. Comments

It seems important to emphasize that only hair roots in the anagen (proliferative) phase of the growth cycle are susceptible to the chemical and physical influences dealt with in this account. Hairs in the telogen (resting) phase of the cycle fail to show any detectable morphological changes following these agents, and there is no evidence that they fall out sooner than they normally would The failure of telogen hairs to be affected accounts for the fact that hair loss following these agents is apparent on the scalp alone, where most hairs are in anagen. The amount of hair left on the scalp after hair fall has actively occurred is an indication of the proportion of hairs in telogen at the time of exposure Similarly, little or no loss of hair is apparent on the eyebrow following these agents, since most hairs here are normally in telogen

Responses of the anagen hair root to the influences enumerated herein may be divided into two general types. The induction of catagen, and conversion to telogen, is one type. This transition and conversion occurs normally, but in man involves only a small proportion of the population of scalp hairs at one time. The conversion of most anagen hairs to telogen, as may occur following fevers, illness, and pregnancy for example, may be regarded as an exaggerated physiological response On the other hand, the induction of alterations in the normal structure of the anagen hair root, as occurs following the other influences, has no normal counterpart, and constitutes another type of response The definitive factors operating upon and within the hair root in each case would seem to be different. These factors are, however, largely unknown

The hair root, in having phases of metabolic activity and quiescence

wherein responsiveness to various influences depends upon the phase involved, is not unique in biological systems. The phenomenon may have applicability to unsettled questions involving reactions of other tissues in man.

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CHAPTER 19

A Reconsideration of the Phenomenon of Hair Neogenesis, With Particular Reference to the Healing of Cutaneous Wounds in Adult Mammals

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I. INTRODUCTION

Most authorities believe that the complement of hair follicles that a mammal is born with or that it acquires soon after birth is not subsequently reinforced by the production of new follicles (Wolbach, 1951, Rawles, 1955). The individual follicles, however, are almost phoenixlike entities that show alternating phases of activity and inactivity, with accompanying gross structural changes during the hair growth cycle. The purpose of this contribution is to present a critical review of some of the evidence bearing upon the question of the neogenesis of hair follicles in relation to healing wounds in adult skin and in intact, normal adult skin

As a baseline, a brief account of the changes undergone by a hair follicle during its normal growth cycle will be presented and this will be followed by a consideration, in some detail, of the capacity of experimentally damaged follicles to regenerate from their various components, or their capacity for regulation in the terminology of experimental embryology. Information on this latter subject derives from studies on the influence of a variety of treatments on skin which include the topical application of carcinogens, irradiation, freezing *in situ*, and transplantation.

II. Cyclical Degeneration and Regeneration in the Normal Follicle

The events underlying the cyclical activity of a hair follicle, first worked out by Dry (1926), have subsequently been investigated in great detail, notably by Chase (1954a, 1955) and by Montagna (1956) who have related them to cyclical changes in the whole integument. The only epidermal portion of a follicle that is normally permanent is the sebaceous glands and their ducts and the apical part, or neck, of the follicle between the openings of these ducts and the surface of the skin

On completion of its growth phase, mitotic activity in the matrix of a follicle ceases and degenerative changes follow which lead to the establishment of a quiescent follicle containing a club hair. The bulb and lower portion of the external root sheath become reduced to a column of epidermal cells. Most of the cells in this column, which include pigment-forming cells or melanocytes derived from the original melanocyte population of the now degenerated matrix (Butcher, 1951), degenerate and there is a great shortening of the follicle so that the encapsulated club—the keratinized, brushlike anchoring base of the hair shaft—lies just below the level of the sebaceous gland. The surviving cells of the hair stem, which include melanocytes, constitute the "germ" which is continuous with the upper external root sheath. The dermal papilla, now represented by a compact group of small, densely staining cells, sometimes referred to as the papilla "rest," becomes free of its hitherto almost, but not quite, complete investment of epidermal cells Only distally does it remain in contact with the hair germ. The formation of a new hair from the germ and papilla rest is essentially a repetition of the initial embryonic process which resulted in the formation of the first hair from the follicle. The persistence of the dermal papilla during the resting phase, and its participation in the formation of the succeeding hair, remained for long a controversial question. Hertwig (1892) was of the opinion that a new papilla was formed at each succession

Apart from the neck region of a follicle, the only other permanent element from one hair generation to the next is the collagenous connective tissue sheath or dermal basket around the upper part of the follicle [illegible]

from the epidermal cells of the outer root sheath by the "glassy membrane," regarded as a homolog of the basement membrane of the

superficial epidermis The proximal end of the arrector pili muscle is attached to this sheath. This sheath may play an important role in the formation of the club hair (Chase and Montagna, 1954), and is far from static, it undergoes cyclical changes in length, thickness, and staining properties. The fact that the hairs which regenerate in a graft that has been removed, rotated through 180°, and replaced in its donor site, preserve their original graft-specific direction of slope, may be held to give strong support to the concept that the connective tissue sheaths are permanent structures.

III. The Regenerative Capacity of Experimentally Damaged Follicles

A. *Hair Regeneration After the Application of Carcinogens*

Attempts to appraise the significance of the many studies on the action of carcinogens on skin are complicated by the complete neglect on the part of authors for the existence of hair growth cycles and their wavelike progression over the skin in most rodents (Wolbach, 1951, Chase, 1954a). From his own extensive investigations of the effects of single and of repeated applications of 3,4-benzpyrene and 20-methylcholanthrene on murine skin, Wolbach (1951) drew attention to the differential results obtained by applying carcinogens at different stages of the hair growth cycle. Applications during active growth are less injurious than those made during the resting stage (Andreasen and Engelbreth-Holm, 1953) Complete destruction of the sebaceous glands follows within 4 days of a single painting of mouse skin with 0.6% methylcholanthrene in benzene (Simpson and Cramer, 1943, 1945), whereas the superficial epidermis, apparently less susceptible, merely undergoes hyperplasia. Complete regeneration of these glands occurs from cells of the outer root sheath as soon as there is active hair growth in the follicles (Montagna and Chase, 1950) When hair development is arrested or delayed, however, e g by x-irradiation, new sebaceous glands fail to develop until hair development recurs (Chase and Montagna, 1951).

Complete necrosis of the epidermis and other epithelial structures occurs within 7 days of a single topical application to mouse skin of a 0.5% solution of 9,10-dimethyl-1,2-benzanthracene in acetone (Orr, 1955). Resurfacing of the lesions takes place by centripetal migration of epithelium from the skin at the margins. On the basis of careful histological studies of the repair process, it has been claimed that hair follicles and sebaceous glands are reconstituted by differentiation *de novo* from downgrowths of the new superficial epidermis into the pre-

existing dermal baskets of the old follicles if still available (Orr, 1955). If the treatment destroys the architectural pattern of the superficial dermis, regeneration of the hair follicles and their glands no longer occurs Gillmann *et al.* (1955a, b) have also championed the cause of hair neogenesis from their findings on the effects of repeated paintings of murine skin with methylcholanthrene. This caused an almost complete destruction of the hair follicles and sebaceous glands and damaged the papillary layer of the dermis. They state that, during the epidermal hyperplasia following this trauma, rete pegs invade the dermis and "may be induced to form new follicles with re-epilation, if the underlying tissue can respond by the neogenesis of hair papillae." According to them, if gross alteration of the dermis has occurred then hair formation ceases, the epidermal downgrowths failing to form hairs. In both of the above studies, in the complete absence of evidence as to the fate of the dermal papillae, which very probably survived, the validity of the claims to have demonstrated the *de novo* formation of hair follicles must be questioned

For some inexplicable reason nearly everyone who has studied epidermal carcinogenesis in the mouse has used albino stocks. Not only has this greatly obscured the very existence and progress of hair growth cycles—in pigmented mice the activity of the follicles is revealed by a conspicuous deep-seated leaden-blue color of the skin, inactivity being indicated by a pinkish-white color—it has also precluded the possibility of obtaining useful information concerning the fate of the melanocytes, and made it practically impossible to follow the fate of the papillae by virtue of their accumulation of residual pigment (Dry, 1926).

More relevant to the problem of hair neogenesis is the work of Silberberg and Silberberg (1947) who made a quantitative study of the effect of repeated applications of 20-methylcholanthrene in benzene on guinea pigs' skin This treatment did not cause necrosis and resulted in an increase in the number of hair follicles This increase posed the question whether it merely represented accelerated growth and replacement within the *same* follicles, or whether it was indicative of the formation of *new* follicles The authors believed that many of the epidermal pegs or cords, which apparently arose from the superficial epithelium and which showed small club-shaped thickenings at their dermal ends, might represent new hairs being formed, the new dermal papillae developing under the influence of the epidermal downgrowths They suggested that under ordinary conditions new papillae do not develop in postnatal life, the influence of potent growth stimuli such as polycyclic hydrocarbons being essential

B. *Hair Regeneration After Irradiation*

Hair follicles are more susceptible to damage from x-irradiation than the superficial epidermis, the cells of the matrix of the growing follicle being among the most sensitive elements of the skin to the damaging effects of ionizing radiations. The dermal papillae, on the other hand, are relatively resistant (Geary, 1952; Chase, 1954b).

Growing follicles are more sensitive to irradiation than resting ones (Chase and Montagna, 1951; Geary, 1952). In the mouse, exposure of a growing follicle to 300 r causes the hair to fall out within a few days Its matrix undergoes degenerative changes and the follicle enters the resting phase prematurely without the formation of a club hair and without undergoing the usual shortening in length which normally brings its base almost up to the level of the sebaceous gland. Eventually shortening does occur, and then the dermal papilla induces the formation of a new bulb. A dosage of 1500 r will cause loss of the hairs and their failure to regenerate on account of damage to the papilla With the more resistant resting follicles, a dosage of the order of 1500 r is required to cause even a temporary epilation. This seems to be caused by damage or destruction of the epidermal elements responsible for anchoring the club hair (Geary, 1952) The melanocytes of the hair germs and matrices are susceptible to x-rays, their destruction resulting in the production of depigmented hairs Other things being equal, for a given dosage of radiation, destruction of these cells is more complete in a resting than in an active follicle (Chase, 1954a)

Complete necrosis of both the superficial and the follicular epidermis follows within about 10–20 days of the exposure of mouse skin to a dosage of 5000 r (Argyris, 1954) The lesion is made good by ingrowth of epithelium from the neighboring undamaged superficial epidermis and the external root sheath of follicles

The action of ultraviolet light on the skin of newborn mice was investigated by Lacassagne and Laterjet (1946), who found that a dosage of 1500 finsens caused necrosis of the full thickness of the skin down to the panniculus During the healing of the lesions the ingrowing epithelium tended to dip down into the underlying connective tissue Although most of these epidermal downgrowths degenerated, a few, so it is stated, found conditions suitable for their differentiation into new follicles Here, as with the experiments cited on the action of carcinogens on skin, there is no evidence of destruction of the dermal papillae which may have been responsible for the production of the new hairs.

C. *Hair Regeneration After Freezing Skin* in Situ

The general conclusion that may be drawn from the work considered so far, namely that follicles can regenerate completely provided that the dermal papillae survive and eventually make contact with epidermal cells again, is strengthened by Taylor's findings (1949) on the action of intense local cooling of the skin of rats *in situ*. He applied solid carbon dioxide (temperature —79°C) to skin areas bearing pigmented hairs. Although there was a marked differential susceptibility of the melanocytes in follicles that were active at the time of freezing, this *differential was not demonstrable with resting follicles. Rapid freezing* of the skin by contact with solid CO_2 for 5 seconds caused only temporary edema but resulted in the failure of a large number of follicles to produce hairs, and consequently a sparser growth in the frozen area. There was no interruption in the activity of the follicles which survived. When contact with the refrigerant was extended to 20 seconds, however, there was complete devitalization of the full thickness of the skin and of the underlying panniculus carnosus. Eventually, through undermining by ingrowing epithelium from the wound margins, the eschar sloughed, leaving a thin, bald layer of skin without a muscle layer and without follicles. Within this area some hairs began to appear after a long delay but they were invariably white in color and, at first, fine and short Taylor very reasonably attributes the slow re-epilation of these areas to the *de novo* formation of hair follicles, independent of old, preexisting follicle rudiments He considers that *de novo* formation of hair buds from the basal layer of regenerated epidermis is an *inductive* process which "occurs only when the tissue underlying the new epithelium is old dermis or after newly formed dermis has become fully differentiated."

D *The Regeneration of Follicles After Transplantation of Skin*

In the early healing-in phase of a skin *autograft*, i.e. a graft transplanted to the same individual from which it was removed, there is an amoeboid upward migration of the entire follicular epithelium toward the surface and a dilatation of the follicle openings. The tremendously enhanced *mitotic activity of the superficial epidermis around the fourth* to eighth postoperative days, accompanied by this migratory activity of the follicular epithelium, culminates in a throwing-off or shedding of all the orignal hairs of the graft which are trapped in the thick cuticle or "ghost" shed by the superficial epidermis The graft surface becomes indented with cone- or flask-shaped epithelialized pits at the sites of former follicle openings, and at the bases of these pits lie the acini of the sebaceous glands Regeneration of the follicles takes place

within a few days of the shedding of the original hairs so that from about the fourteenth day onward, fine new hairs begin to pierce the surface. Eventually, provided that the graft was not too thick, and that it was transplanted to an adequately vascular bed, so that there was no ischemic necrosis, the graft regenerates a replica of its previous hair crop, faithful with respect to its orientation, the arrangement of its follicles into clusters, and its pigmentation. This healing-in phase of a skin graft was first described in detail in the rabbit (Medawar, 1944). Subsequently it has been found that the events are very similar in other mammals (Billingham and Medawar, 1951). Despite the great transient upheavals which normally occur in skin grafts, the new hairs are indubitably generated within the *original* connective tissue sheaths under the influence of the *original* dermal papillae

If a skin autograft is transplanted in such a way that its revascularization is greatly delayed, as for example, when the full thickness of the skin, with its fatty layer and panniculus still intact, is transplanted to a poorly vascular bed, then the graft no longer regains its *status quo ante*. The hairs in such a graft usually remain in place for a long time, being trapped in the superficial layers of the dermis which ulcerate, become necrotic, and give rise to a firm eschar. Eventually, after a matter of weeks, the eschar is undermined by epithelium from the host skin at the wound margins and is sloughed off. Hairs may appear again but their orientation is usually random and, in the mouse, they are usually depigmented. This is evidence that melanocytes in the hair germs have been destroyed (Werder and Hardin, 1955). Unlike that of the guinea pig, the superficial epidermis of rats, mice, and rabbits possesses only a very sparse complement of melanocytes Consequently, although the Malpighian-type cells of the follicles can be replaced by those originating from the new graft epithelium, melanocytes are not normally so replaced Pepper's work (1956) with melanocyte suspensions indicates that repigmentation of the hair would probably take place if the melanocytes were forthcoming from the superficial epidermis The disorientation of the hairs is evidence of the irreparable damage to the fibrous endoskeleton of the graft. Many of the dermal papillae probably survive, being situated fairly deep in the graft corium, they should be less severely exposed to the ischemic conditions prevailing at more superficial levels. For want of evidence, it is prudent to assume that the hairs on these grafts are of regenerative origin. These experiments emphasize the amazing capacity of follicles for regulation or reconstruction. The reappearance of fingerprints following attempts to efface them by scraping off the epidermis or destroying it with corrosive agents points to essentially the same thing.

A question still unresolved is whether anatomical preservation of the fibrous connective tissue follicle sheaths, in the absence of viable papillae, can result in the production of new hairs. The speculations of Weiss (1944, 1950) might encourage one to believe this to be the case. Two lines of evidence having some bearing upon this question will now be considered

If skin is transplanted between different individuals of the same species the "foreign" cells in the homografts provoke an immunological reaction on the part of the host, the so-called homograft reaction. After a severe vascular inflammatory reaction within the graft, its entire cellular population is destroyed, leaving its collagenous endoskeleton virtually intact (Medawar, 1944). Under the appropriate conditions this collagen pad becomes overgrown by epidermis from the host, while at the same time some new collagen fibers are laid down within it by invasive host fibroblasts. Columns of epithelial cells that appear to be new hair follicle primordia grow down into the old follicle sheaths. These "follicles," however, are aborted and the undersurface of the superficial epidermis assumes a plane configuration. The "foreign" dermis becomes progressively avascular and disappears after a few months. If, instead of transplanting skin homografts to hitherto unsensitized recipients, they are transplanted to animals which have previously reacted against skin from the same donor, the second set of grafts is destroyed more rapidly and without the intervention of an inflammatory upheaval The result is a much better anatomical preservation of the dermis, with its fibrous follicle sheaths, than in the case of first-set grafts. Despite these considerations, however, their collagenous remains also fail to produce hairs when they become re-epithelialized (Medawar, 1945)

In the course of studies on wound contracture, Billingham and Russell (unpublished) froze dried grafts of full-thickness skin removed from the dorsa of rabbits' ears and then, after reconstitution in excess normal saline, transplanted the "devitalized" autologous skin to vascular beds prepared in the skin of the thoracic wall. Despite the fact that the collagenous remnants of the grafts were re-epithelialized and were of autologous origin, they were gradually resorbed and no hairs appeared

Although these studies with devitalized grafts are inconclusive, they provide no indication that the preservation of the fibrous frameworks of follicle sheaths is sufficient to "mold" downgrowing epidermis to differentiate into proper follicles in the absence of viable papillae. No direct experiment has yet been performed in a mammal to prove whether the papilla rudiment alone, after complete destruction or removal of the

native epidermal cell population of a follicle, is capable of interacting with subsequent epidermal downgrowths to regenerate a normal follicle This might be practicable with some of the larger vibrissae. The soundness of the concept is suggested from the analyses of the regeneration of feathers in adult birds (Lillie and Wang, 1941, 1944; Wang, 1943) The papilla is an indispensable component of the feather and exercises an inductive influence on its covering layer of epidermis during feather regeneration, the regional type of feather which is produced is determined by the integrity of the epidermis. In any consideration of the indispensability, or otherwise of the integrity of the connective tissue sheath for follicle regeneration, heed must be taken of the view that the causative defect in genetically hairless mice (*hrhr*) lies in the follicle sheath (Chase, 1954c).

IV. Hair Regeneration from Healed Full-Thickness Wounds in Rabbits' Skin

In the rabbit the natural healing of experimental lesions produced by the removal of the full thickness of the skin down to, or even through, the panniculus, is the outcome of two distinct, but largely simultaneous processes that provide, in turn, a temporary and a definitive repair During the first week or two the defect becomes filled with highly vascular granulation tissue which is gradually surfaced by the ingrowth of epithelium from the wound margins. The conclusion of the temporary repair is marked by the closure of the raw wound surface by epithelium Definitive repair is achieved by contracture, a forced inward movement of the wound margins in response to tensile forces generated within the granulation tissue "fill" of the wound, or its derivatives (Abercrombie *et al*, 1954) This provides an ephemeral "organ of contracture" (Billingham and Russell, 1956a).

Because of contracture, it is normally impossible to maintain an extensive area of epithelialized granulation tissue for more than a week or two. In an ingenious attempt to obtain an uninterrupted sheet of skin epithelium in the rabbit, Breedis (1954) prevented contracture of circular, full-thickness excision wounds by means of stainless steel splints. Scattered diffuse thickenings appeared in the epithelium which grew in over the wound From these, by about the thirtieth postoperative day, there developed downward projections into the granulation tissue which was undergoing a fibrous transformation. These projections were identifiable as incipient hair follicles By about the fortieth day, recognizable follicles appeared in great numbers.

These findings of Breedis have now been confirmed and extended by Billingham and Russell (1956a, b) during the course of a quantitative analysis of the phenomenon of wound contracture in rabbit skin.

It was found that contracture in the dressed wounds 20 to 40 cm^2 in area took place at a constant specific rate, ranging from 5 to 8% of the wound area per day. In about 60% of the animals contracture proceeded so far that only a narrow linear scar remained, the wound as such having been completely eliminated through approximation of its original margins. In the remainder of the animals, however, for some inexplicable reason, contracture ceased after the wounds had been reduced in extent to about 5 cm^2 and their size thereafter was more or less constant and



FIG 1 An extensive crop of white hairs of new formation growing in the scar tissue of an incompletely contracted wound prepared 70 days previously by removal of the full thickness of the skin from an extensive rectangular area on the side of the chest of an adult brown agouti rabbit. Magnification, × 1

their margins incisive From about the fortieth day onward, commencing at the periphery, the smooth epithelial surfaces of these wounds began to be pierced by fine hairs. With the production of hairs, the wounds became transformed into a sort of *ad hoc* skin (Figs. 1 and 2) In no sense, however, did this hair-bearing scar tissue resemble normal skin. It remained chronically edematous and slightly inflamed—hence its conspicuous pink color when the hair was clipped—for at least 3 months, and probably for much longer. It was much thicker than normal skin and had a soft, cheesy consistency when cut and its collagen fibers were disposed mainly in a horizontal plane instead of in the three-

dimensional packing characteristic of skin. It could be transplanted, when its hairs were shed and subsequently regenerated.

Normal skin is never properly regenerated in any type of wound in which the dermis is involved. In man (Gillman *et al.*, 1955c, Gillman and Penn, 1956) the trivial wound represented by the donor site of a Thiersch graft never regains its original morphology, even after many years In the healed site not only does the anatomy of the dermoepidermal junction differ from that of normal skin, but also the morphology



FIG 2 Transverse section through the hair-bearing scar tissue of an incompletely contracted wound of 70 days standing Magnification × 45

of the new connective tissue which replaces the lost dermis differs considerably from that of true dermis, although it is not scar tissue

In the rabbit wounds, despite the fact that nearly all animals were of pigmented breeds, the new hairs were invariably white in color (Billingham and Russell, 1956b). Their orientation bore no constant relationship to that of the neighboring normal fur, and they tended to slope outward from the center of the wound Their density of distribution, however, was normal and they were grouped together into small follicle clusters Guard hairs were not identified. Well-developed sebaceous glands were present and the hairs were medullated Apart from their lack of pigment, the only other anomaly of these hairs was the absence of arrectores pilorum muscles

As with Breedis' experiments, it is practically inconceivable that any of the bases of the native hair follicle population could have been left behind when the wounds were prepared; the follicle bases in pigmented breeds of rabbits are easily identifiable. Moreover, the vast number of hairs present in such wounds (up to 3500), makes a postulated origin from pre-existing follicle rudiments completely untenable. In his careful study of the histogenesis of these hairs Breedis found that, by the time the epithelial buds were identifiable as incipient follicles, they already had dermal papillae beneath them so that it was impossible to determine whether or not one structure induced the other. Histological studies on the development of vibrissae indicate that the papilla rudiment may be the first to appear (Rawles, 1955). In very young chick embryos the mesoderm establishes where feathers shall form (Cairns and Saunders, 1954), when embryonic mouse skin is cultivated *in vitro* no epidermal thickenings form in the absence of mesoderm and no dermal papillae can be found in areas from which the epidermis has been removed (Hardy, 1951). There can be little doubt that an interaction of epidermis and dermis is involved in initiating the development of hairs, and the process of hair neogenesis, as seen in wounds in adult rabbits, probably does not differ significantly from that which occurs normally in neonatal life.

That the phenomenon of hair neogenesis in rabbits' skin has only recently been discovered is explicable partly in terms of the high proportion of wounds which undergo complete contracture and partly because several weeks must elapse before the hairs appear Furthermore, the majority of previous studies on wound healing and even on skin grafting have not entailed the application of dressings Contracture under these circumstances takes place too quickly and too completely for the phenomenon to reveal itself. The formation of a thick layer of granulation tissue may be an essential prerequisite for hair neogenesis in rabbits (Van der Brenk, 1956) In a transparent chamber technique it is possible to follow the regeneration of epithelium in wounds prepared by removing the full thickness of the skin from rabbits' ears. In such cases regeneration of hairs does not take place

The only other mammal in which hair neogenesis has been reported in healed full-thickness skin wounds is the rat. Incipient development of new hair follicles has been observed in healing undressed full-thickness wounds of 21 days' standing (Dann *et al*, 1941). Lindquist (1946), who carried out a very comprehensive study of the healing of skin defects in rats under a variety of conditions, did not observe this phenomenon.

V. Does Hair Neogenesis Occur During Normal Growth?

The most obvious and long standing evidence that new follicles are not laid down *after* the pilary system has developed in early life, derives from histological studies on skin and depends upon the fact that follicles differentiating *de novo* have never been convincingly demonstrated. Moreover, from what is known about hair neogenesis in rabbits' skin wounds, it might be anticipated that hairs of new formation in normal skin would be white in color in all species in which there is a poorly developed system of melanocytes in the superficial epidermis and that they would be devoid of arrectores pilorum muscles Yet, save possibly in extreme old age, the coats of animals do not undergo a progressive dilution with white hairs.

The more elaborate hypothesis that, as an animal grows in size and its skin surface expands, new follicles might be formed insidiously, thus preventing a growth dilution of the initial follicle complement, becomes exceedingly difficult to sustain in the light of the following evidence Szabo (1957), making use of Boyd's estimates (1935) of the changes in surface area undergone by various parts of the human body from the time of birth to the age of 24 years, has shown that the actual differential densities of distribution of hair follicles and sweat glands in different regions of the body of an adult are exactly what one would expect on the assumption that they were evenly distributed throughout the integument initially.

Billingham and Medawar (1955) have studied the fate of "islands" of intact skin or grafts left behind at the centers of large raw areas prepared on the sides of rabbits' chests from which the full thickness of the skin has been removed down to the panniculus By this artifice the tensile forces generated by the granulation tissue which subsequently filled the wounds were concentrated on the central skin islands or grafts. In response to these forces their areas increased by a factor of about 4 During this expansion new collagen was laid down in relation to the pre-existing fibrous structure and the grafts maintained their normal thickness due to a process of intussusceptive growth The mechanism of the expansion brought about in these "islands," or grafts, is probably the same as that responsible for the ordinary growth of the skin. Grafts transplanted from adult to very young animals are known to expand with the growth of their host in a similar manner In Billingham and Medawar's experiments no new follicle clusters were formed, and the original ones became separated from their neighbors by a distance roughly proportional to the linear enlargement of the graft

VI. Hair Neogenesis and the Growth of Antlers

The antlers of deer are deciduous bony growths which are shed annually, on renewal they may form progressively more branches or tines as the animal grows older. In the Virginia deer (*Odocoileus virginianus*) in its natural habitat the antlers begin to grow in April or May and reach maturity in August. The mature antlers, consisting of bare, dead bone, remain firmly attached in place until midwinter, when shedding occurs. The particular importance of antlers for the present purpose lies in the fact that from the time they begin to develop until they reach maturity they are covered with a layer of hair-bearing skin, the "velvet." When growth of the antlers is complete, the velvet becomes necrotic, probably as a consequence of interference with its blood supply (Waldo *et al*, 1949), and detaches itself from the mature antlers some months before they are shed.

After the antlers have been shed, each frontal bone presents an antler pedicle whose free "raw" distal end is slightly concave. This concavity becomes partially filled with what appears to be granulation or fibrocellular tissue (Macewen, 1920, Wislocki, 1942). Epidermis from the surrounding skin grows in over the defect left by the base of the shed antler. The distal surface of the pedicle remains smooth and bald at first, but later downy hairs appear. At the time of growth, the pedicles elongate at a phenomenal rate and tines, or lateral processes, are budded off the main beams. The rate of growth is prodigious; within 3 to 4 months the antlers of elk and caribou reach lengths of about 5 feet, corresponding to daily increases in length of 1.5 to 2 cm. The growing antler consists of a bony core which is surrounded by a layer of periosteum and skin. Structurally the latter does not bear the slightest resemblance to scar tissue, it contains all the elements of cervine skin (Noback and Modell, 1928) although it is much thinner than that covering the skull (Macewen, 1920). From the velvet relatively short, soft hairs emerge at right angles as opposed to the coarse hairs covering the forehead. These hairs are pigmented and have well-developed sebaceous glands. This skin contains neither sweat glands nor arrectores pilorum muscles associated with these follicles. In the wound-healing experiments of Billingham and Russell (1956a, b) the hairs of neogenic origin also lacked arrectores pilorum muscles.

Although the corium of the velvet lacks a typical subcutaneous layer, there is, in its place, a dense layer of parallel bundles of collagenous tissue in which are located the main blood vessels supplying the antler. This layer has been considered to be a highly modified subcutaneous lamina (Wislocki, 1942) rather than the outer layer of the periosteum

of the antler. At the tips of the developing beams and, later, at the tips of the tines are the germinal caps from which the entire antlers differentiate. These germinal caps bear more than a superficial resemblance to the blastemas from which amputated limbs regenerate in lower vertebrates. Macewen (1920) drew an analogy between the growth of an antler after the shedding of its predecessor with that of a lizard's tail after amputation.

In these germinal regions the velvet is firmly united to the periosteal bed and fewer and more delicate hairs are present than elsewhere on the antlers Growth of the entire antler seems to be dependent upon, and controlled by, the formation of a germinal periosteal bed derived from the skin and, in this sense, "growth of the antlers depends upon the organizing influence of the skin" (Wislocki, 1942).

This regeneration of a considerable expanse of new, hair-bearing skin each year is a unique achievement among mammals. At present the mechanism is practically unknown, although the antler cycle itself is hormonally controlled (Wislocki, 1956). Intussusceptive growth of skin overlying the growing bony pedicles on the frontal bones of the skull clearly does not account for the enormous areas of integument involved: in elk and caribou the velvet may account for at least 20% of the total skin surface of the body If intussusceptive growth were responsible, one would expect deer to become progressively more bald year by year as the follicles on their heads become farther and farther apart Baldness is not an affliction of the deer The only possible explanation of the facts is that neogenesis of *both* skin and follicles takes place year by year The whole problem awaits further elucidation and its final analysis may be of the utmost importance for the light it sheds on wound healing, the origin of melanocytes in the follicles of new formation and, the embryogenesis of a complex organ

Obviously the velvet derives from cellular ingredients present in the skin which lies over the original bony protuberance of the frontal bones. When the antler rudiment of a one-year-old red deer was grafted from its normal position to the nasofrontal surface of the frontal bone, an antler grew, showing that the competence of skin to form velvet is not rigidly circumscribed to a small area of cranial skin (Jaczewski, 1956)

It seemed fitting to conclude this review by citing, in some detail, the *experimentum crucis* on hair neogenesis and on skin regeneration performed by nature in a group of adult mammals long before the phenomenon was accidentally revealed on a small scale by laboratory artifice. Even in the absence of any experimental evidence, had some of the older authorities consulted their zoologically informed colleagues,

the possibility of hair neogenesis might never have been dismissed so vehemently. For once a heresy has been vindicated.

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CHAPTER 20

Ageing of the Human Male Scalp[1]

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I. INTRODUCTION

Studies of the capillaries that surround the hair follicles (Montagna and Ellis, 1957), sebaceous glands, and sweat glands (Ellis and Montagna, 1958), have called attention to some of the striking changes that occur during the ageing process in the human scalp. These observations have led to a comprehensive survey of the human male scalp from birth to senescence, and it will be shown that remarkable changes occur in the cutaneous structures of the scalp and their accompanying blood vessels.

Certain ageing changes in the human male scalp, such as the receding hairline, the gradual thinning and graying of the hair, and, frequently, the partial or complete balding are quite apparent These are changes that involve the hair follicles and are the result of interactions between the male sex hormones and the genetic constitution of the individual (Hamilton, 1942). The epidermis and its underlying capillary bed also undergo profound changes, the sebaceous glands become larger, while the eccrine sweat glands are modified more subtly. All these alterations involve concommitant changes in the capillaries that accompany these structures

II ARTERIAL CIRCULATION IN THE SCALP

To understand the changes that take place in the capillary beds during ageing, it is necessary to review first the arterial supply to the scalp. Of the five arterial plexuses formed in the scalp, only three are re-

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lated to the vascularity of the epidermis and the cutaneous appendages The terminology of these three plexuses is unnecessarily confusing, and Durward and Rudall (1958) suggest that the terms subepidermal, dermal, and hypodermal be used in identifying them. These authors equate the subepidermal plexus with the capillary loops which supply the epidermis, the dermal plexus lies just beneath this, and the hypodermal plexus is located deep in the dermis

At the earliest stages of their development, all of the cutaneous appendages are supplied by blood vessels from the dermal plexus (Spalteholtz, 1927) However, as the appendages increase in length and penetrate deeper into the dermis, their lower parts become invested by vessels branching up from the hypodermal plexus. The capillary bed around hairs and sweat glands, then, is derived from two sources: their upper parts are supplied by branches of the dermal plexus and their lower parts by vessels stemming from the hypodermal plexus. The sebaceous glands and the arrectores pilorum muscles may also receive their blood supply from both the dermal and hypodermal plexuses. The papillary body of the dermis which interdigitates with the rete pegs of the epidermis, is supplied by the capillaries of the subepidermal plexus, formed from superficial branches of the dermal plexus during the development of the scalp

The vessels of the subepidermal and dermal plexuses have the same diameter as capillaries do, and their endothelium is rich in alkaline phosphatase The hypodermal plexus consists of arterioles with numerous side branches which may supply hair follicles, sebaceous glands, arrectores pilorum muscles, and eccrine sweat glands. This system of trunk arterioles sends branches to more cutaneous appendages than do any of the plexuses, and has an endothelium which is usually reactive for alkaline phosphatase

Three plexuses can be demonstrated in the scalp of young adult men In Fig 1, a section through the scalp of a 26-year-old man, the arrow points to an arteriole of the hypodermal plexus at the junction of the fatty hypodermis (H) and the dense dermis (D). Branches from the hypodermal plexus supply the lower parts of the growing hair follicles which are embedded in the hypodermis Several trunk arterioles connecting the hypodermal and dermal plexuses can be seen giving off side branches to the sweat glands and the sebaceous glands The loops of the subepidermal plexus are seen just beneath the epidermis (E), the branches at the base of the subepidermal loops are part of the dermal plexus In the bald scalp of a 67-year-old man (Fig. 2), most of the vascular architecture of the scalp has vanished Vestiges of the sub-



FIG 1 Thick vertical section through the scalp of a 26-year-old man The epidermis (E), dermis (D), and hypodermis (H) are indicated A resting and parts of several growing hair follicles are shown, and blood vessels of the subepidermal, dermal, and hypodermal (arrow) plexuses are visible Alkaline phosphatase, light carmalum counterstain Magnification × 15



FIG 2 A similar section of the bald scalp of a 67-year-old man The dermis is thinner and only lanugo hairs are present, eccrine sweat glands and sebaceous glands are abundant in the dermis The blood vessels in the scalp are reduced greatly in numbe
Alkaline phosphatase, light [illegible]

epidermal and dermal plexuses form shallow arches under the epidermis, and only the sebaceous glands and the eccrine sweat glands have a rich capillary bed. Although the hypodermal plexus is not apparent, the hypodermal fat is penetrated by numerous capillary twigs. These two illustrations, then, demonstrate that the circulatory patterns of the scalp undergo profound changes with increasing age.

III. Ageing Changes in the Scalp

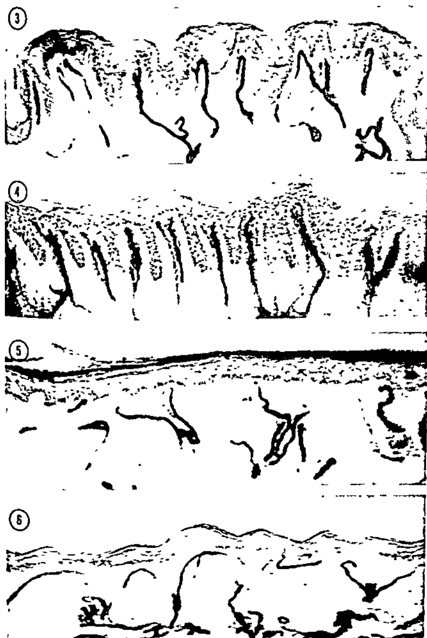
Specimens of scalp obtained from subjects 2 to 6 hours after death are entirely satisfactory for demonstrating alkaline phosphatase activity. Scalp was always taken from the same region, close to the vertex; only specimens of male subjects, ranging from birth to 88 years in age, were used. After fixation for 4 hours in cold, unbuffered 10% neutral formalin, thick frozen sections were cut, some tangential and others vertical to the surface of the epidermis. Thin sections are less useful because they show only fragments of capillaries, in thick sections capillaries can frequently be traced from their parent arteriole to the structure they are supplying. The sections were processed through the Gomori (1952) azo-dye technique for alkaline phosphatase. The endothelium of the capillaries of the skin stains a conspicuous blue-black, indicating strong enzyme activity. Even blocked or collapsed capillaries, which would be difficult to demonstrate with other techniques, are visualized here. Since the capillaries and the terminal arterioles are almost the only structures in the scalp which contain the enzyme, they appear black against a pale or unstained background (Montagna and Ellis, 1957, Ellis and Montagna, 1958).

To examine the details of the ageing changes in the capillary circulation, each class of cutaneous structure in the scalp will be considered separately.

A. The Epidermis

The epidermis of the scalp of young men consists of an outer thick stratum corneum, a thick stratum spinosum, and the generative layer, or stratum basale. The stratum basale and stratum spinosum are thrown into folds, the rete pegs, which protrude into the dermis.

With ageing, the thick epidermis of youth, with its deep rete pegs changes to a thinner, flatter layer, the rete pegs are reduced to mere ripples or they may completely disappear. In the subepidermal circulation, the number of capillary loops under the epidermis become greatly reduced, and the individual loops become shorter. Finally, in senescence, the capillary loops of the subepidermal plexus almost completely disappear (Chiale, 1927). These changes can be seen in Figs. 3–6



Figs 3–6 Ageing changes in the epidermis of the male scalp and the subepidermal capillaries Alkaline phosphatase Magnifications × 108 Fig 3 Three-year-old boy Fig 4 Thirty-three-year-old man Fig 5 Nonbald 66-year-old man Fig 6 Bald man, 68-years old

In Fig 3, a vertical section through the scalp of a 3-year-old boy, the epidermis is thick and thrown into folds. This is the characteristic appearance of the skin of infants and young children. The rete pegs of the epidermis are well established and long capillary loops penetrate the papillae of the dermis which are thrust up among the rete pegs. These capillary loops are close together, and provide a rich blood supply to the overlying epidermis Both growing and vellous hair follicles were present in other parts of this specimen.

Figure 4 shows a portion of the scalp of a 33-year-old man. Growing hairs were predominant in this specimen. The rete pegs or epidermal papillae which dovetail with the dermis at regular intervals are developed maximally. The total thickness of the epidermis is somewhat greater than that in Fig. 3. The capillary loops are evenly spaced beneath the epidermis, they are fewer in number and shorter than those in Fig 3.

The pattern of capillaries beneath the epidermis of the scalp from a 66-year-old man is shown in Fig. 5 This subject was not bald, but had thinning grey hair many of which were in the resting phase. The epidermis is thinner than in either Figs. 3 or 4. The rete pegs have nearly disappeared and are identifiable only as shallow ridges which make the dermal-epidermal junction slightly uneven. In some regions, even such vestiges of the rete pegs are lost Low, short capillary loops are found at irregular intervals beneath the epidermis. The total number of the capillary loops is considerably diminished, however, around the pilary canals the rete pegs may be relatively unchanged and resemble those shown in Fig 4. In such places the capillary beds remain similar to those found in younger skin

Figure 6 is a longitudinal section through the bald scalp of a man 68 years old, in which only vellous hairs were present. The epidermis is distinctly thinner than in any of the three preceding figures and the rete pegs have been ironed out, flattening the dermal-epidermal junction A suggestion of rete pegs remains around the orifices of sweat glands and the pilary canal of lanugo hairs. Some capillary loops may also be present near these structures. As can be seen in this figure, the capillaries supplying the epidermis are shallow and very sparse. Instead of loops they form low arcades underneath the epidermis and the subepidermal plexus is nearly obliterated

A better impression of the ageing changes in the epidermis and its capillary bed may be obtained from tangential sections through the dermal-epidermal junction Figure 7 shows such a tangential section through the scalp of an infant 10 months old The rete pegs form a



FIGS 7–9 Tangential sections through the dermal-epidermal junction, showing the ageing changes in the subepidermal plexus and in the rete pegs Alkaline phosphatase Magnifications × 54 Fig 7 Infant 10 months old Fig 8 Twenty-six-year-old man Fig 9 Nonbald 65-year-old man

reticulum which surrounds islands of pars papillaris. The capillary twigs near the center of each dermal papilla connect with branches beneath the rete pegs and together they form the subepidermal plexus. Although this specimen has many features of Fig. 3, the capillary loops are not as well developed. In contrast with this, the scalp of the newborn child has scarcely formed rete pegs, the epidermis is thin, and the capillary bed at the dermal-epidermal junction lacks a definitive pattern.

In Fig. 8, a tangential section through the scalp of a 26-year-old man, the capillary loops between the rete pegs is richly developed The scalp of this subject agrees in most respects with the longitudinal view of the scalp of the 33-year-old man shown in Fig. 4. The true nature of the rete pegs is revealed, showing that they are, in fact, a network of epidermal ridges surrounding islands of dermis. The dermal-epidermal junction is developed to its peak of complexity, and consists of a multitude of protruding dermal papillae which fit into epidermal sockets ringed by the rete pegs Each dermal papilla contains a well-developed capillary loop, which, at a slightly lower level in the dermis, joins the subepidermal plexus The capillary loops are numerous and evenly spaced Each pilary canal is surrounded by a circle of six to eight capillary loops Capillaries are also particularly abundant around the orifices of the eccrine sweat glands. At the left in Fig 8, and at a deeper level, the limbs of the capillary loops which connect with the dermal plexus may be seen. The rich vascularization of the scalps of this age provides the epidermis with an abundant blood supply.

The differences between Fig. 9, a tangential section through the scalp of a man 65 years old, and Fig. 8 are extraordinary. The rete pegs are almost absent, and the capillary beds, even those around the hair follicles are greatly reduced The pattern of evenly spaced capillaries found in the infant (Fig 7) and in the young adult (Fig. 8) has completely disappeared, leaving only a few blood vessels that bear little relationship to the dermal and epidermal structures. The epidermis is very thin. The deeper-lying blood vessels in the center of the figure, are few in number and show little organization In place of the rich capillary bed found at the dermal-epidermal junction of the scalp of young men, there are only a few disorganized branches from the dermal plexus Under the epidermis, rudiments of capillary loops are all that remain of the subepidermal plexus

It is probable that the deterioration of the subepidermal plexus with ageing may be responsible for the changes that occur in the epidermis. Katzberg (1957) also noted the gradual thinning of the epidermis and a decrease in the frequency and the size of the rete pegs with ageing,

resulting in a general decrease in the surface area at the dermal-epidermal junction He believes that there also occurs an increase in the mitotic activity and in the desquamation of the epidermis, with a decrease in the life span of the epidermal cells. These observations, however, were not made on the scalp. Since the respiratory quotient (RQ) of the skin decreases in old age (Walter and Amersback, 1948), it is not easy to reconcile the increase in mitotic activity found in the epidermis by Katzberg (1957). Mitotic activity in the epidermis is dependent upon an adequate supply of glucose and oxygen (Bullough, 1952) The scant subepidermal circulation in the aged scalp cannot provide a very rich supply of nutrients to the epidermis The loss of rete pegs results in a considerable decrease in surface area at the dermal-epidermal junction, and this might be considered to be a compensatory factor. All of these factors indicate, then, that the alterations in the structure of the epidermis are direct results of the decreased circulation in the subepidermal plexus.

The cause of the circulatory changes are not known There is a distinct correlation between the presence of hair in the scalp and the integrity of the subepidermal plexus Even the scalps of very old subjects have remnants of the subepidermal plexus around growing hair follicles Perhaps the same endocrine and genetic factors which control hair growth also have an effect on the subepidermal circulation of the scalp The sebaceous glands and eccrine sweat glands, which seem to be resistant to these changes, may be under different control.

B *Hair Follicles*

Hair follicles and their adnexa are arranged in groups which are embedded in the dense connective tissue of the dermis (Koelliker, 1889). The distribution of hair groups has been studied in the fetus by Kato (1936) and Fleischhauer (1953), but changes in hair groups with ageing, have not been described in the scalp before

Hair groups are clearly recognized in the newborn child and are easily seen in the adult, but with increasing age the demarcations between then becomes less evident (Figs 10–12) It appears that there is a reduction in the number of hair follicles in each group, especially during early postnatal growth This may result from a separation of the hair groups by the spreading and stretching of the scalp in growth. The loss of demarcations between the hair groups in older scalps is caused by the divergence of individual hair follicles and the enlargement of the sebaceous glands associated with the hair follicle (Fig 12).

Changes also occur in the types of hairs found within the hair groups.



FIGS 10–12 Tangential sections of scalps at the level of the sebaceous [gl]ands Changes in the hair groups and in the development of the sebaceous glands [ar]e seen with increasing age Alkaline phosphatase, light carmalum counterstain [M]agnifications × 72 Fig 10 Infant of 8 months Fig 11 Thirty-year-old [m]an Fig 12 Man 68-year-old

The hair groups of infants contain one to three large growing hair follicles and a variable number of follicles of lanugo hairs. The latter can be identified easily in phosphatase preparations by the intense enzymatic activity in the bulb of the follicle (Figs. 10, 15). In the non-balding scalp of young or middle-aged men, hair groups are definite and contain one to four actively growing large hairs. Lanugo hairs and resting hairs may occasionally be present (Figs. 1, 11) The scalps of older men with thinning hair or partial baldness have ill-defined hair groups. These scalps contain mostly resting and lanugo hairs with some growing hairs (Fig. 12). In totally bald scalps, hair groups are indetectable and all of the follicles are of the lanugo type.

The circulatory patterns around growing and resting hair follicles have been described by Montagna and Ellis (1957). The lower third of active hair follicles is enveloped by a rich vascular plexus composed of long, more or less parallel vessels connected by cross-shunts. The parallel vessels, which are assumed to be terminal arterioles, are of wider bore than the cross-shunts and are directly connected to the hypodermal plexus Some of these parallel vessels enter the papilla pore and supply the dermal papilla. Most of the cross-shunts drop out just above the level of the bulb, and up to the level of the sebaceous glands the follicle is surrounded only by the palisade of parallel longitudinal vessels. At the level of the sebaceous glands the vessels once again form a network that envelops both the glands and the pilary canal. The parallel vessels lose their identity around the pilary canal and terminate in a loosely woven network that extends to the surface of the skin Capillaries from both the dermal and epidermal plexuses may also contribute to the plexus surrounding the pilary canal at this level Quiescent hair follicles have no bulb and they are much shorter than active ones (Fig 1) A dense pallisade of capillaries connected by short, transverse vessels surrounds the free dermal papilla and extends for a distance below it A few straight vessels from this bundle rise along the sides of the follicle, go up to the level of the sebaceous glands, and form a network like the one described in active follicles. A few cross-shunts form a sparse network at the base of the epithelial capsule around the club hair

In contrast, the lanugo hairs have a scant papillary supply, generally consisting of only one or two vessels associated with the hair bulb These capillaries may stem from the dermal plexus or the trunk arterioles connecting the dermal and hypodermal plexuses A hair of this type is shown in Fig 15

Remarkable vascular changes also take place within the hair groups

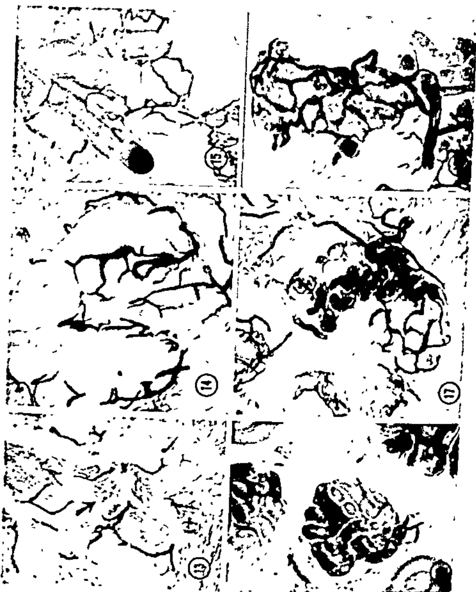

15
14
17
13

FIG 13 Photomicrograph showing the small sebaceous glands (arrow) and the capillaries around them in the scalp of a boy 3 years old Alkaline phosphatase Magnification: × 54.

FIG 14 Sebaceous glands and their capillary network in a man 33 years old. Alkaline phosphatase Magnification. × 108

FIG 15 Multilobular sebaceous glands associated with a lanugo hair follicle in the scalp of a bald man 72 years old Some of the capillaries interconnect with an eccrine sweat gland below (lower left). Alkaline phosphatase Magnification· × 54.

FIG 16 Coiled eccrine sweat gland in the scalp of a newborn child The secretory portion of the coiled tubule is rich in alkaline phosphatase Alkaline phosphatase with light carmalum counterstain Magnification × 54

FIG 17 Coiled portions of an eccrine sweat gland in the scalp of a nonbald 66-year-old man. The secretory portion of the gland is rich in alkaline phosphatase, and the coiled duct, which is nonreactive, is also closely invested with capillaries Alkaline phosphatase Magnification· × 54

FIG 18 A branch of the hypodermal plexus forms an intricate capillary bed for a coiled eccrine sweat gland in the scalp of a 67-year-old man There is almost no alkaline phosphatase in the secretory portion of the tubule Alkaline phosphatase, light carmalum counterstain Magnification: × 60

In the scalp of infants the majority of the capillaries are associated with the hair follicles and there is little development of the sebaceous glands (Fig. 10). In the mature scalp the sebaceous glands are surrounded by a rich network of capillaries (Fig. 11). In the aged scalp, the capillary circulation around the sebaceous glands achieves primary importance and is much more extensive than that associated with the hair follicles (Fig 12).

C. The Sebaceous Glands

In the scalp of infants, the sebaceous glands are small (Fig. 10). In a three-year-old boy, the sebaceous glands are present as tiny lobules (arrow) at the side of the upper part of the hair follicle (Fig. 13). Only two or three short capillaries arising from the vessels of the hair follicle surround the small sebaceous glands of boys. In young men, the sebaceous glands are considerably larger, and consist of one to three large lobules (Figs. 11, 14). The entire gland is surrounded by capillaries that are somewhat evenly spaced and which closely follow the contours of the gland. In the aged nonbalding scalp the sebaceous glands are generally large, showing as many as six lobules in a single section (Fig 12) Capillaries encompass each lobule and are frequently embedded in the delicate connective tissue partitions between adjacent lobules The characteristic orientation of the sebaceous gland on one side of the hair follicle (Figs 10, 11), so typical of younger scalps, becomes obscure As already noted, the enlargement of the sebaceous glands in scalps of this age is primarily responsible for the lack of demarcations between hair groups

In the bald scalps the sebaceous glands are much larger and consist of many lobules They are larger in some individuals than they are in others of a similar age In every case, however, the sebaceous glands are the most conspicuous organs in the scalp A rich capillary plexus invests these glands, covering the duct and acinar portions and penetrates the crypts between the lobules (Fig 15). The capillary circulation may come directly from the hypodermal arterial plexus, from neighboring lanugo hair follicles, or from nearby eccrine sweat glands An increasing disorganization in the blood vascular system of the scalp seems to bring about these ageing changes.

The variations in the size of the sebaceous glands in subjects of the same age makes it difficult to observe minor ageing changes However, certain major changes are notable The sudden increase in the size and activity of the sebaceous glands with the onset of puberty is well known (Miescher and Schonberg 1942) The capillary bed of the glands,

which is derived from the vessels supplying the hair follicles, is also augmented at that time. Although it has been reported that the fat content of human hair does not diminish with ageing (Nicolaides and Rothman, 1953), and that there is no significant change in the sebaceous glands which are correlative with old age (Ejiri, 1937), the present study shows that with ageing the sebaceous glands actually increase in size, become lobular, and acquire a richer capillary bed In the bald scalp the sebaceous glands are one of the few structures which retain a rich capillary bed. The genetic and hormonal factors which wipe out the capillary bed of the epidermis and have such overwhelming effects on the hair follicles, do not seem to affect the sebaceous glands or their capillary supply.

D. The Eccrine Sweat Glands

The eccrine sweat glands of the scalp are well developed at birth (Fig 16). Each gland is a simple tubule that consists of a basal coiled segment, and a straight duct that opens onto the epidermis In the newborn, the coiled segment lies precisely at the level of the hypodermal plexus and derives its capillary supply from these vessels In succeeding years, the dermis thickens but the coils of the glands remain stationary and eventually come to lie in the middle of the dermis. With this change, their capillary supply comes to be derived principally from the arterial trunks that connect the dermal and hypodermal plexuses Some of these vessels accompany the straight duct all the way to the epidermis and anastomose with the vessels of the dermal and subepidermal plexuses In aged individuals, the derivation of the capillary bed is less clear-cut. Capillaries surrounding the coiled portion of the eccrine sweat gland may anastomose with the blood vessels surrounding adjacent hair follicles and sebaceous glands (Fig. 15), as well as with vessels of the hypodermal plexus (Figs. 17, 18) and branches of the dermal plexus

Although there are individual variations in the length of the coiled tubules of the eccrine sweat glands, the length of the coiled tube increases with age At any rate, the sweat glands of the scalp do not atrophy with ageing, and their capillary bed seems to become even richer (Figs 16–18)

In young people the secretory segment of the glands is consistently rich in alkaline phosphatase (Fig 16), and can be readily distinguished from the coiled duct which shows no enzymatic activity. Although these two segments have many structural differences (Montagna, 1957), they are equally well supplied with capillaries (Ellis and Montagna,

1958) (Figs. 17, 18). With increasing age, the content of alkaline phosphatase in the secretory portion of the tubules becomes variable, and in some the enzyme seems to be lacking entirely (Figs. 17, 18). The capillaries surrounding them, however, remain reactive.

Like the sebaceous glands, eccrine sweat glands do not become atrophic with ageing, and may even become larger. In spite of the reduction in the general vascular supply of the scalp with ageing (Fig 2) the blood supply to the eccrine glands may actually be augmented. Thus, eccrine sweat glands, like the sebaceous glands, are not affected *by the same hormonal and genetic factors that control hair growth*, the structure of the epidermis, and the subepidermal arterial plexus.

IV. Summary

Ageing changes in the human male scalp have been studied from birth to senescence using an alkaline phosphatase technique on thick frozen sections.

With ageing, the epidermis becomes thin and the dermal-epidermal junction flattens; the capillary loops supplying the epidermis disappear and the subepidermal plexus deteriorates.

The hair follicles which are aggregated into groups in infants and young men become separated in aged scalps, and in bald scalps there are no distinct hair groups. With ageing there is a progressive transformation of growing hair follicles into lanugo types. The growing hair follicles are richly vascularized but the lanugo hairs have only one or two capillaries associated with their hair bulb.

The sebaceous glands increase in size with ageing and become multilobular; they have a rich capillary bed even in bald scalps. There are no signs of atrophy in ageing eccrine sweat glands; they may even become larger with a richer capillary plexus The abundant alkaline phosphatase activity which is always present in the secretory coils of the eccrine sweat glands of infants and young adults becomes variable with ageing

The same hormonal and genetic factors which control the growth *of the hair follicles* in the male scalp are correlated *with the deterioration* of the subepidermal plexus and the accompanying ageing changes in the epidermis. The eccrine sweat glands and the sebaceous glands are resistant to ageing changes and are not affected by the endocrine and genetic influences that produce balding.

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CHAPTER 21

Summary

William Montagna

Hair follicles are simple organs composed of cells that are mostly alike, this simplicity, however, is deceptive The cells of hair follicles arise from the common pool of the matrix and do a variety of similar, but subtly different things to form the hair. The precision of the periods of growth and rest, the coordinated processes for the formation of different keratins in cells that are in contact with each other, the rigorous distribution of pigment in some cells and not in others, the energy requirements for growth and differentiation, and the effects of physical and chemical factors on the growth of hair are now better understood than they have been before. With this knowledge at hand, the understanding of the prime factors that guide and control hair growth cannot be far off.

Hair follicles originate in the embryo from focal proliferations of the basal cells of the epidermis. The anlagen of pilary complexes are composed of ectodermal and mesodermal elements from the very beginning, and the follicles perish if this association is interrupted. Fetal hair follicles contain functioning melanocytes throughout the bulb, the inner root sheath, and the basal layer of the outer root sheath, in the adult follicle, however, visible pigment cells are found only in the upper bulb, and in the cortex and medulla of the hair. When the embryonic follicle is becoming organized, the first keratinized structure formed is the tip of the inner root sheath, which is conical As the follicle grows longer, it adds more inner root sheath from behind, and the hair is formed inside the inner sheath When the follicle has attained its definitive length, the inner root sheath and hair move up toward the surface, but the inner sheath becomes fragmented in the pilary canal and the hair emerges alone Mesenchymal cells in the corium become aligned and form the bundles of arrectores pilorum muscles, which become attached to the bulge of the outer sheath secondarily. None of the early fetal hair follicles is vascularized As the follicles become thicker and produce larger hairs, they become surrounded by capillary nets, and the dermal papillae are invaded by capillary tufts

In man, the population of hair follicles is much denser in the fetus than it is in the adult. The regional distribution of follicles over the entire body of the early fetus is similar, although there are more follicles in the head than elsewhere As the body grows and takes shape, hair follicles are spaced farther apart in the trunk and the extremities

than they are in the head. The variations become most marked during postnatal life, and in the adult the head has about three times as many hair follicles as the trunk; the arms and legs have the least. These regional variations must be brought about by the different rates of growth of the body surface. In spite of gross differences, there is no significant difference between the distribution of hair in the skin of men and women

To understand the function of a hair follicle, one must know its structure Misunderstandings can be avoided when one has a concrete idea of what hair follicles really look like. Felix Pinkus understood this so well that he spent much of his precious time painstakingly reconstructing three-dimensional models of cutaneous appendages from serial sections. The impressive series of models reconstructed by Van Scott shows that although the basic structure of all human hair follicles is similar, there exist minor modifications and deviations from this pattern that can serve to identify the hairs from the different regions. For example, the large hair follicles from the scalp occur singly or in groups, those in groups remain separate units up to just below the epidermis, at which point they become joined in a common follicular pore from which several hairs emerge. The hair follicles of the male beard occur singly, the pilary canal of these follicles is characteristically divided into two channels: the hair passes through one of the channels, the excretory duct of the sebaceous gland opens through the other. In the upper back, approximately one-half of the hair follicles are in groups of two, conjoined at the pilary canal.

The proliferative part of a hair follicle is a small region in the lower half of the bulb, the matrix, where indifferent cells divide, move up the bulb in orderly rows, and differentiate into hair and the inner root sheath The outer root sheath, formed in the early stages of the growth cycle, remains fairly static The undifferentiated cells that have arisen from the matrix move into the upper part of the bulb, where they become larger and undergo differentiation. The cuticle of the inner sheath is interlocked with that of the hair, and hair and inner sheath must grow at the same rate In the upper part of the bulb, melanocytes form melanin and feed it out only to the presumptive cells of the cortex and medulla of the hair, the cuticle of the hair and the entire inner sheath remain nonpigmented When a follicle approaches the end of its growth cycle, a club hair is formed above the bulb and the bulb is largely destroyed, leaving the follicle much shorter, and having a hair germ of undifferentiated cells, which is the seed for the next generation of hair. When activity is set off again, the simple hair germ rebuilds a bulb which then manufactures hair and inner root sheath again

Active hair follicles are rich sources of chemical substances, many of which have been studied with histochemical methods. The findings of histochemistry are interesting and often suggestive, but thus far they constitute only an accumulation of facts; these are steadily attaining significance. A number of inorganic substances, among them calcium, magnesium, zinc, sulfates, phosphate, and iron are present in different amounts in quiescent and active follicles. Glycogen, PAS-positive, but diastase-resistant material, and acid mucopolysaccharides are much more abundant in and around growing hair follicles than in resting ones. We now have a clear understanding of the histotopography of phospholipids, unsaturated lipids, and plasmal, but the significance of these is unknown Amino acids, protein-bound sulfhydryl groups and disulfide groups, as well as nucleic acids, have characteristic distributions during different stages of hair growth. Hair follicles contain many enzymes. It is premature to venture interpretations of the significance of phosphorylase, succinic dehydrogenase, cytochrome oxidase, esterases, acid and alkaline phosphatases, 5-nucleotidase, glucose-6-phosphatase, cholinesterase, β-glucuronidase, aminopeptidase, and carbonic anhydrase in hair follicles, but these enzymes all have a precise distribution in growing and quiescent follicles. Histochemical studies of hair keratins are still hampered by the technical limitations of the available methods. These methods and others will be refined and the chemical composition of hair follicles is slowly unfolding.

Under the electron microscope, keratinizing epidermal tissues are seen to be separated from the dermis by a continuous, structureless membrane about 400 A The epidermal cells in the basal layer are attached to this dermo-epidermal membrane, but their plasma membranes are separated from it by a less dense layer. Epidermal cells contain small mitochondria and agranular vesicles comprising the Golgi bodies. They also contain large numbers of ribonucleoprotein particles, which unlike those in protein-secreting cells, do not form an endoplasmic reticulum Where adjacent cells touch at the nodes of Bizzozero, there is a plate consisting of a thickened cell membrane and several submicroscopic layers of dense material within the cytoplasm Keratin fibrils sprout in tufts from these plates

Fibrous keratin first appears in epidermal cells as wispy bundles of fine filaments, in the differentiating cells of the hair these filaments [illegible]

[illegible]

between the filaments In high resolution electron micrographs, fibrous keratin appears to consist of a system of fine parallel α-filaments about

60 A in diameter, held together by the γ-component, a cement substance high in cystine and probably not fibrous.

Trichohyalin granules are precursors of keratin. After these granules have accumulated in the cells of the inner root sheath, they transform into fibrils, which are different from those that form in the cortex of the hair, and form a keratin different from that of the hair cortex.

The formation of many fibrous systems is preceded by the synthesis of a nonfibrous macromolecular precursor, this is transformed into fibrous protofibrils, arranged into more organized structures, and finally the product is hardened or tanned. Cells that form fibroprotein have dense ribonucleoprotein (RNP) particles in their cytoplasm If the fiber-precursor is to be secreted from the cell, the RNP particles are associated with an endoplasmic reticulum; if the protein fibrils accumulate within the cells, as in the hair, the RNP particles lie freely in clusters in the cytoplasm In fiber formation the nonfibrous precursor is transformed into protofibrils by the formation of linear or helical aggregates in which the original structure of the macromolecule is preserved The organization of protofibrils into parallel arrays, networks, and membranes is due to some added control mechanism, such as shear due to flow, or the presence of already organized material. Keratin protofibrils form spontaneously, in hair follicles they seem to owe their orientation to a slight initial flow in the deformed cells, this controls the direction of the fibrils which are added subsequently.

In the early stages of keratinization, the formation of cytoplasmic fibrils is a basic mechanism which later becomes associated with the *decomposition and elimination of certain cytoplasmic and nuclear elements* Although these are common properties of keratinizing cells of both hair cortex and epidermis, the mechanism of keratinization must be different in the two structures In the differentiating cells of the hair cortex, the cytoplasmic fibrils gradually reach such a high concentration that the cells consist of practically all fibrils. At this point, nuclear and cytoplasmic activities cease, and the nonkeratin constituents are almost completely eliminated In differentiating epidermal cells the formation of fibrils is less gradual, and the fibrils never seem to reach such high concentrations, also, the elimination of nuclear and cytoplasmic nonkeratin components is less complete

Hair keratin and keratin formed by the epidermis, have different quantities of amino acids While hair keratin contains histidine, lysine, and arginine in the characteristic ratio of 1:3 10, this ratio is different in epidermal keratin Matoltsy suggests that either the availability of amino acids is different in the hair follicle and the epidermis, or that

keratin synthesis proceeds according to different principles in these tissues.

The relation of the vascular patterns to the cycles of growth has been largely overlooked. Peripheral blood vessels are small and difficult to interpret in histological material, but by injecting whole animals with India ink, Durward and Rudall, and Ryder have visualized the major networks and plexuses that accompany the hair follicles of the rat, rabbit, and the lamb. In the pelage of the rat the monotrich has a rich network of capillaries around the lower half of the follicle, and the dermal papilla contains a tuft of capillaries. Awls have a less dense plexus of capillaries around the follicle and no capillaries in the papilla; the smaller follicles are not surrounded by an individual plexus, and their papillae are not vascularized. Naturally occurring hair growth waves in the rat and the rabbit are accompanied by a corresponding intensification of the blood supply, which keeps step with the growth or regression of the follicles.

Capitalizing on the fact that the endothelium of capillaries is strongly reactive for alkaline phosphatase, capillary networks can be demonstrated clearly around hair follicles in thick sections of skin. In man, the capillary plexus around large follicles, like that around the monotrichs of rats, is densest around the bulb of growing follicles. The rest of the follicle is surrounded only by a few arterioles and capillaries. The dermal papilla of large active follicles has a large capillary plexus. In man, as in the rat, rabbit, and lamb, progressively smaller follicles have scantier capillary plexuses; the very small follicles of vellus hairs have practically no plexus, and the dermal papillae are avascular. During the transition from active state to quiescence, the bulb largely degenerates and the follicle withdraws from the capillary plexus, which remains intact but collapsed at the base of the follicle. The dermal papilla also withdraws from its plexus. These observations do not provide an answer to the question whether the initiation of growth in a follicle is preceded by an engorgement of capillaries or accompanied by it, but evidence favors the latter view.

Some follicles on the human body are surrounded by nerves, they may be found anywhere on the body but are more numerous on the scalp and face, and in the perineum. These follicles are surrounded by a plexus of nerves just below the entrance of the sebaceous glands into the pilary canal, the lower part of the follicle has no demonstrable nerves. All of these nerves, like those that surround the sinus hair follicles of other mammals, contain specific cholinesterase and are probably sensory nerves.

Until now nothing had been known about the energy

for mitosis in the cells of the matrix. Bullough and Lawrence, applying to the hair follicles of mice the same methods which they have used to study the surface epidermis, have opened a new field of research. Unlike the surface epidermis, the cells of the matrix have no diurnal rhythms, and no depression of mitosis occurs after forced exercise. In starved mice, mitotic activity does not become depressed in the matrix cells until after the animals are in a state of collapse, even then, the addition of glucose restores mitotic activity to normal. Full shock almost completely inhibits mitotic activity of the matrix cells, but partial shock does not greatly affect it. When skin from fully shocked mice is incubated with glucose, mitotic activity in the matrix returns to normal almost immediately.

Active mitosis in a hair bulb requires adequate supplies of oxygen and of some suitable carbohydrate substrate, in the absence of either, mitotic activity is powerfully inhibited. Glucose, fructose, and pyruvate are ideal substrates for the support of mitotic activity; the various Krebs cycle intermediates tested are not efficient. Mitotic activity in hair bulbs is inhibited by any substance that inhibits the process of glycolysis, the Krebs cycle, or the cytochrome system; 2,4-dinitrophenol, which inhibits the process of energy transfer, has the same effect.

The high mitotic activity in a hair bulb can only be maintained by a high level of energy produced in the cells. Therefore, it must be expected that mitotically active hair bulbs will normally absorb large quantities of glucose and oxygen; this has been shown to occur with the use of radioactive glucose.

The germ of quiescent follicles has two centers of growth one in contact with the dermal papilla and giving rise to the bulb, the other alongside it and producing the elongated lower external sheath The cells of the matrix have great power to organize themselves, even when they are mechanically displaced they give rise to the separate layers of the inner sheath and the hair shaft.

Melanocytes reside in the upper bulb, capping the dermal appilla There is evidence, however, that some melanocytes, which do not form melanin under normal conditions, are also found in the outer root sheath, as in the embryo When active follicles of the human scalp are irradiated, these quiescent melanocytes reveal their presence by becoming active The follicles of some animals, as, for instance, the seal, normally contain a few small active melanocytes in the outer sheath Melanin granules, being opaque, make a microscopic study of their formation difficult This has been resolved by studies of melanocytes in human hair follicles with the electron microscope These studies have not only shown how melanin granules are formed, but also how

they are transferred from the melanocytes to the cells of the cortex and medulla In the cytoplasm of the melanocyte, a region near the nucleus, that corresponds to the Golgi apparatus, contains numerous vesicles that consist of an outer membrane and several concentric inner ones. Definitive granules are formed by a gradual deposition of melanin upon these vesicles. The amelanotic melanocytes in albino hairs also form these complex vesicles, but pigment is not deposited upon them. The dendritic processes of the melanocytes are insinuated in the intercellular space of the presumptive cells of the cortex and medulla; these cells phagocytose the pigment-containing tips of the dendrites and acquire pigment of their own. This method of pigment transfer seems to occur also in the cells of the epidermis

Hairs have a wide range of color hues, although only black, brown, and yellow pigment granules can be seen in them under the microscope The nature of hair pigments and their possible metabolic pathways are presented here for the first time by Fitzpatrick and his colleagues Black and brown pigments are similar chemically and in the way they are formed; the yellow pigment, pheomelanin, is different.

When DL-tyrosine-2-C^{14} is used as a substrate, tyrosine is incorporated into the pigment cells in the hair bulbs of mice. Tyrosinase activity, however, is closely related to the stages of the hair cycle In C^{57} black mice, tyrosinase activity is not detected with this radioautographic technique during anagen I and II; it appears weakly in anagen III and gradually increases in amount during anagen IV, V, and VI. Enzyme activity is absent during telogen. The ability to oxidize tyrosine is greater in melanic than in pheomelanic follicles The hair follicles of albino mice show no uptake of labeled tyrosine.

Tyrosinase seems to be the natural precursor of melanin Although pheomelanic hair follicles utilize tyrosinase as a substrate *in vitro*, the pigment formed is abnormal, and the oxidation of tyrosine may be involved only indirectly in the formation of this pigment The activity of the genes in producing pheomelanin in the guinea pig and mouse is very definite, and there are no intermediates between melanin and pheomelanin This clear-cut action of the genes suggests the presence of a "switch-mechanism," probably involving one enzymatic step Two things suggest that tyrosine and tryptophan intermediates have a dual role in this switch-mechanism: the formation of the red-yellow pigment, xanthommatin, which is dependent on the conversion of dopa to dopa quinone in the presence of tyrosinase, and the nonenzymatic oxidation of 3-hydroxykynurenine to xanthommatin by the dopa quinone, can be reduced back to dopa There is no melanin formed in pheomelanic hair follicles of man and guinea pig, when skin is incubated in

dopa 3-hydroxykynurenine in a molar ratio of 1.4 for 20 hours. If the ratio of dopa:3-hydroxykynurenine is 4:1, no black pigment is formed in 4 hours, but black pigment is deposited after 20 hours, when all the 3-hydroxykynurenine has been oxidized. The formation of pheomelanin could, then, be the result of the oxidation of an *o*-aminophenol by dopa quinone, produced by the oxidation action of tyrosinase or dopa. This is compatible with the observation that pheomelanic hair follicles contain tyrosinase, and it would explain the pigmentary switch-mechanism leading to either melanin or pheomelanin, depending on the absence or presence of *o*-aminophenol. The critical enzyme operating the switch could be one bringing about the hydroxylation of an aromatic amine. Pheomelanic hair emits an orange or yellow fluorescent light under near-ultraviolet light The fluorescence of a mixture of 3-hydroxykynurenine and dopa in which hair follicles have been incubated resembles that of an alkaline extract of pheomelanic hair.

The literature abounds in reports on the effects of nutritional deficiencies on the growth of hair These reports, however, have limited value, since most investigators have paid little attention to the normal cycles of growth of the hairs in their experimental animals. Ryder's report in this book on the growth of the wool of sheep takes into account many of the factors that could influence hair growth. Since species differences occur, his observations pertain only to the sheep, and must be applied with caution to the hair growth in other animals

The weight of wool that a sheep grows is controlled by the amount and quality of the food available. A poor diet reduces the breaking strength, and the length and diameter of wool fibers, but the reinforcement of the diet with both protein and carbohydrate corrects these defects and increases wool production Carbohydrate is needed to provide energy for protein utilization, and for the release of protein for wool formation Carbohydrate is also an essential requirement for mitotic activity in hair follicles, as shown by Bullough and Lawrence.

Cystine or methionine, while essential for hair growth in many animals, are not needed by the sheep, which can synthesize cystine from sulfate B vitamins are necessary agents for the growth of hair, and pantothenic acid seems to be associated with the utilization of copper Deficiencies of copper causes a loss of pigment in hair, and in wool, a loss of the crimp Copper is believed to catalyze the oxidation of SH— to —S—S— groups, although it has not been possible to detect copper in the follicles either with histochemical methods or with the use of Cu^{64}

Within a few minutes after the injection of cystine labeled with S^{35}, radioactive particles appear first in the outer root sheath above the

bulb, this substance must enter the follicle at this level from the surrounding capillary net, and not through the vessels of the papilla. Soon after an injection of glucose labeled with C^{14}, radioactive particles are recovered in the bulb and not above it, showing that they have entered largely through capillaries of the papilla. These observations give meaning to the plexuses of capillaries in the papilla and around the bulb.

No less confusing than the effects of nutrition are the reports on the effects of hormones on hair growth Statements that a certain hormone "accelerates" or "retards" growth are meaningless unless one first understands normal growth This problem is rendered more difficult by the existence of species differences For example, cortisone compounds initiate hair growth in alopecia areata in the scalp of man, but suppress hair growth in the rat Most of our information at the present time comes from observations on the effects of hormones on hair growth in the rat This animal is eminently suited for such studies since its hair growth cycles are short and precise

In the rat spontaneous growth begins periodically in the belly and spreads dorsally as a wave, when club hairs are plucked from an area, the follicles all grow synchronously The cycle of growth of a follicle is about 26 days, regardless of how activity is initiated. At the end of 26 days, growth ceases, and the follicles become quiescent. Male rats have coarser hair than females, and the skin of the male is covered with flakes of oxidized lipids. Spontaneous growth waves in females lag behind those of males, but the cycle of growth in each follicle is the same in both sexes. After gonadectomy, sex differences disappear; the fur is intermediate in texture between that of males and females, and spontaneous growth resembles that of normal males

Daily treatment with estrogen retards the initiation and the rate of both spontaneous and induced hair growth in animals that have been gonadectomized, adrenalectomized, hypophysectomized, or rendered deficient in thyroid hormone Estrogen induces the growth of fine, sparse hair in all animals except those which have been hypophysectomized Daily treatment with androgen has no apparent effect on hair growth except that it promotes a coarse pelage in all except hypophysectomized rats.

Spontaneous replacement of hair is noticeably retarded during pregnancy and lactation, although induced growth by plucking is normal, hair growth is transiently accelerated when the young are removed from the mother. These effects are not duplicated by treating intact females with progesterone, but are partially simulated when nursing females are treated with luteotropic hormone

Adrenalectomy accelerates the initiation and the spread of growth waves, but has no effect on the rate of growth of the individual follicles. Adrenalectomy has no effect on induced growth, and the pelage regrown is unaffected. Daily treatment with small doses of cortisone inhibits the spontaneous initiation of hair growth in intact, gonadectomized, or adrenalectomized rats, but has no effect on follicles already growing. Large doses of cortisone completely inhibit hair growth in intact rats, but have no effect on follicles already growing. All hair growth is inhibited when propylthiouracil-treated or hypophysectomized animals are injected with small doses of cortisone. In all of these cases growth commences as soon as the cortisone is discontinued. Daily treatment with deoxycorticosterone has no effect on hair growth in intact or adrenalectomized rats.

Continuous treatment with adrenaline inhibits spontaneous hair growth in intact animals and delays the response to plucking, but growth proceeds normally once it has started. Prolonged treatment with adrenaline produces a local inhibition of spontaneous or induced growth. These effects are neither mediated nor potentiated by the thyroid. They are, however, partially linked to adrenocortical activity. Adrenaline inhibits hair growth more in cortisone-treated, adrenalectomized rats than in adrenalectomized animals not receiving cortisone; the effects are not due to the cortisone.

Spontaneous growth is markedly retarded in alloxan-diabetic animals, but after an initial delay, induced growth is normal Treatment with phlorhizin does not affect hair growth despite a continued glycosuria and hypoglycemia. Insulin restores spontaneous replacement to normal in alloxan-diabetic animals, and enhances growth in intact animals despite the low level of glucose it produces in the blood. Glucose-treated intact animals, on the other hand, have normal regrowth after plucking, but their spontaneous growth is often retarded. Insulin, *then, seems to be more directly involved in hair growth than glucose,* perhaps by regulating the utilization of glucose from the blood during the early stages of follicle growth.

An intake of propylthiouracil that produces a deficiency in thyroid hormone inhibits the spontaneous waves of hair growth. Induced growth, however, is practically normal. Injections of thyroxine accelerate spontaneous replacement of hair in propylthiouracil-treated rats and in normal rats, the cycle of growth, however, remains normal regardless of how activity is initiated Thyroxine and cortisone have antagonistic effects on hair growth, and one hormone can be used to offset the effects of the other. Such a relationship does not exist between thyroxine and gonadal hormones.

Hypophysectomy, which accelerates the initiation and spread of spontaneous growth waves, has no effect on the rate of growth, the cycle of growth is normal after plucking, but the pelage is infantile. The administration of ACTH inhibits hair growth in intact, gonadectomized, and hypophysectomized rats, but has no effect on that of adrenalectomized animals. This inhibition is obviously mediated through the adrenal cortex. The pituitary also exerts a restraint on hair growth by means of the adrenal cortex. Hypophysectomy removes this restraint.

Implants of pituitary tissue or injections of growth hormone restore the pelage of hypophysectomized rats to an adult texture. Hair remains infantile in hypophysectomized rats even when treated with gonadal hormones. Sex hormones, then, modify the type of hair produced only if growth hormone is present.

The rate of growth of hairs in man is more difficult to assess than that in the rat. Hamilton and his colleagues have devised techniques that provide repeatable quantitative measurements of the rate of growth of hairs in certain regions of the body. With these methods, data have been obtained for each sex throughout the lifespan in Caucasian and Japanese populations. The standards constructed from these values can help in evaluating some aspects of physiologic age, and in studying the endocrine status of the individual.

The growth of the beard in Caucasian males is greater than that in Japanese. In contrast to the high incidence of facial hirsutism in Caucasian women, not a single instance of it was found in Japanese women. Males of both ethnic groups have similar values for the mean diameter of coarse hairs and for the percentage of gray hairs with advancing age Growth of axillary hair is more pronounced in Caucasian than in Japanese men, and much more so in Caucasian than in Japanese women of comparable ages Caucasian males also have a greater tendency to develop coarse hairs on the external ears and to become bald than do Japanese males

Secondary sex characters and certain sex-differing pathologic states fail to develop in men who do not mature sexually. The degree to which maintenance of these conditions, once fully developed, depends upon gonadal secretions, is different, and listed in decreasing order of dependence are the axillary hair, beard, and common baldness. Under ordinary conditions these traits are dependent upon gonadal secretions for development, and in some instances for maintenance. The extent to which these states develop is regulated chiefly by heredity and ageing. Studies of twins and members of large families, supplemented by comparisons of Caucasians and Japanese, delineate and emphasize the large measure of control exerted by genetic factors

Secondary sex characters merge almost indistinguishably with male-selecting conditions like common baldness and severe acne. There are suggestions that this spectrum may extend to some of the more lethal male-selecting pathologic states, and to the shorter duration of life in males than in females in man and other animals In some ways, the control of pilary secondary sex characters seems to be analogous, and may provide clues, to those of certain sex-selecting pathologic states.

During its period of growth, a follicle produces hair to its fullest capacity and cannot be pushed beyond its limits. Increased hair production, then, can only be achieved by initiating activity in quiescent follicles, and preventing them from going into the resting state. Many physical agents that cause enough damage to stimulate a moderate epidermal hyperplasia are effective in initiating growth in quiescent follicles In the human scalp, doses of x-ray high enough to cause severe damage to growing follicles, actually initiate growth in quiescent ones. In the mouse and rabbit, a dose of x-ray of about 1500 r stimulates resting follicles, while damaging growing ones. The most practical and widely used experimental method for initiating growth in follicles is the plucking of club hairs

After epilating doses of x-ray, the bulb of active follicles in the human scalp largely degenerates. X-rays accelerate all the changes of catagen in active follicles, except that a club hair is not formed and the hair falls out. When the degenerative changes have ceased, the follicles remain in telogen for a time, and then grow normally again The effects of ionizing radiation on hair follicles can be studied by examining, under the low power of the microscope, unstained hair roots plucked from the irradiated scalp This method should have useful application in medicine. Changes can be seen clearly two days after irradiation, and become progressively manifest until 10 to 14 days. These changes, which are confined to growing hair follicles, begin in the matrix and progress to the rest of the bulb, until it is completely atrophied At the end of 10 to 14 days, when complete disintegration of the bulb has occurred, the ends of the hairs are tapered, and they fall out at the end of 3 weeks. In a few hairs the roots recover during the first week, assume a structurally normal bulb, and continue to produce a hair, the hair shaft in these follicles has a smaller diameter in a demarcated zone which can be identified, as judged by its distance from the bulb, as that portion of the hair that was produced during the time that the follicle suffered from the effects of the irradiation The percentage of growing hairs showing morphological defects due to irradiation may be calculated by examining one hundred or more hairs pulled from the areas of the scalp exposed to x-rays Such examinations,

repeated at intervals during the week after the irradiation, show that the percentage of damaged hairs increases linearly in relation to both time and to the dose of irradiation to which the scalp was exposed.

Microscopic examination of unstained roots of hairs pulled from the scalp of patients receiving therapeutic doses of amethopterin reveals a transient but reversible injury to the hair bulb. The hair formed during the time of administration of the drug has a smaller diameter, when the administration of the drug is stopped, the bulb again produces a hair of normal diameter. As a result, hairs from patients who have received amethopterin have zones of constrictions. The degree of constriction corresponds to the dose of the drugs employed, and may be so severe that the shaft breaks at this point when the hair is pulled or combed.

A concluding statement must be made on the postnatal development of hair follicles. In the young of mice, rats, and rabbits, which are born naked, many of the hair follicles continue to develop from the epidermis during the first 2 or 3 postnatal days. In adult animals, however, the population of hair follicles is apparently fixed and does not increase. Several investigations on the healing of cutaneous lesions in mice, rats, guinea pigs, rabbits, and even man, provide some evidence that hair follicles can form anew from the adult epidermis. During the healing of extensive cutaneous wounds in rabbits, hair follicles may be formed from the epithelialized scar tissue Such new follicles possess sebaceous glands, but they lack arrectores pilorum muscles and pigment, although the rabbits used may belong to pigmented breeds. This suggests that these hairs are of completely new formation and have not originated from follicle remnants left behind in the wound bed.

Incontestable evidence that completely new follicles can be formed in adult animals is found in the antlers of deer These deciduous bony prongs are shed in midwinter and regenerated during early spring. When growing, they are completely covered by "velvet," a layer of typical hair-bearing skin. When they are fully grown, the skin dries and peels off, leaving the bone core exposed The hairs in the velvet are pigmented and have sebaceous glands but, like the new hairs that grow in the wounds of rabbits, they lack arrectores pilorum muscles Thus, each year throughout its life the deer regenerates a relatively large area of skin, complete with its complement of hair follicles. At least in these cases, then, the rigid view that hair follicles can be formed only in the embryo or the newborn can no longer be sustained.

Lest the optimistic bald reader come to the conclusion that here is hope for the solution of the problem of baldness, let him be warned that baldness and the regrowth of hair just described are very different things Many uncritical observations have been made on baldness.

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20. Somatotropes Hormon

Bereits im Jahre 1921 stellten EVANS und LONG (28) fest, daß Extrakte der Rinderhypophysenvorderlappen bei normalen infantilen Ratten beschleunigtes Wachstum und Gewichtszunahme gegenuber Kontrolltieren hervorrufen. SMITH P. E. fuhrte analoge Versuche bei hypophysektomierten Ratten durch, wo die Unterschiede im Wachstum der Tiere gegenüber den Kontrollen noch ausgeprägter waren. Diese Ergebnisse führten dann zu dem Bestreben, das entsprechende „*Wachstums*"-Hormon aus Hypophysenextrakten rein darzustellen. Später wurde für den isolierten Wachstumsfaktor des Hypophysenvorderlappens die Bezeichnung *somatotropes* Hormon geprägt, die sich allgemein durchsetzte. Das Hormon ist offenbar auch mit dem *diabetogenen* Prinzip der Hypophyse identisch.

Darstellung des Hormons. Zur Isolierung des Hormons wurden mehrere Methoden namentlich aus Rinder-, Schaf- und Schweinedrüsen beschrieben. Zur Extraktion verwendet man zumeist schwach alkalische Losungen und Salzlosungen. Bei der Darstellung ist sorgfältig zu beachten, daß das Hormon ziemlich unbeständig ist. Daher arbeitet man am besten bei Temperaturen bis zu +5° C. Das Hormon hat Globulincharakter und kann mit Salzlosungen von geeigneter Konzentration ausgesalzt werden. Am häufigsten werden hierzu Ammonsulfat oder Natriumchlorid benutzt. Es wurden auch Adsorptionsmethoden zur Reinigung des Hormons angewandt.

LI und EVANS (56, 57) beschrieben 1944 und 1945 ein Verfahren zur Reindarstellung des somatotropen Hormons aus Rinderhypophysen. Die Hypophysenvorderlappen wurden bei —10° C in das Acetontrockenpulver übergefuhrt, und dieses wurde sodann mit Calciumhydroxydlosung vom pH 11,5 extrahiert. Durch Einleiten von CO_2 wurde das pH auf 8,7 herabgesetzt, und nach Abtrennen des Niederschlags wurde die Flussigkeit mit 2 M Ammonsulfatlosung ausgesalzen Der erhaltene Niederschlag wurde nach Lösen in Wasser erneut durch Aussalzen gereinigt; anschließend folgte Dialyse zur Entfernung der Elektrolyte. Wahrend der Dialyse der Losung scheidet sich der Niederschlag des Hormons aus, der nach dem Losen erneut durch Aussalzen bei pH 4,0 mit 0,1 M NaCl-Losung fraktioniert wird; nach Abtrennen des Niederschlags wird mit 5 M NaCl gefallt. Sodann wird die Fraktionierung durch Aussalzen mit Ammonsulfat wiederholt, und nach der Dialyse wird das Hormon im isoelektrischen Punkt pH 6,8—6,9 gefallt. Man wiederholt diese Reinigung noch 2mal und erhalt so ein Somatotropinpräparat, das praktisch von den ubrigen Hypophysenvorderlappenhormonen frei ist.

Fishman (33) beschrieb im Jahre 1947 das Verfahren zur Herstellung eines reinen Somatotropinpräparates, wobei die Extraktion ähnlich wie beim Verfahren von Li und Evans verläuft und die weitere Fraktionierung in der Kälte mit Alkohol vorgenommen wird. Li mit Mitarbeitern (59) stellte schließlich das Hormon in *kristallisierter Form* her. Er ging von dem gereinigten amorphen Präparat aus und benützte Fishmans Methode der Fraktionierung mit Alkohol. Hierbei wurde ständig bei +2° C gearbeitet. In ähnlicher Weise verfuhr auch Wilhelmi mit Mitarbeitern (108) bei der Darstellung des kristallisierten Hormons; dieser veröffentlichte seine Ergebnisse gleichzeitig mit Li im Jahre 1948. Aus 1 kg Rinderdrüsen gewann man 3 g Präparat, das sowohl in der Ultrazentrifuge als auch bei der Elektrophorese homogen war. Neuerdings wurde die modifizierte Methode Wilhelmis zur Darstellung des kristallisierten Somatotropins gemeinsam mit Angaben über die Gewinnung von Somatotropin neben ACTH auch von einem polnischen Autor beschrieben (78).

Neuerdings benutzte Li (65) zur *Darstellung von Somatotropin ein vereinfachtes* Verfahren, bei dem man nach alkalischer Extraktion mit Ammonsulfat aussalzt und aus 1 kg Rindervorderlappen ungefähr 2 g Hormon gewinnt, das sich sowohl *in Reinheitstesten in der Ultrazentrifuge und bei der Elektrophorese als auch in* biologischen Testen als hochwirksam erwies. Alle Isolationsstufen wurden bei Temperaturen unter +1° C ausgeführt. Bei Elektrophorese des Präparats an Stärke bei pH 4,0, 9,0 und 11,2 wurde eine einzige Zone festgestellt (35). Demgegenüber untersuchten Ellis und Evans (25) das Verhalten von nach verschiedenen Verfahren hergestellten Somatotropinpräparaten bei der Elektrophorese und stellten bei allen Proben zwei Eiweißkomponenten fest. Bei der Gegenstromverteilung des Hormons im System mit p-Toluolsulfonsäure und Butanol wurden nur eine Hauptkomponente und verschiedene Mengen einer weiteren Komponente festgestellt (80).

Reid beschäftigte sich in zahlreichen Arbeiten mit Versuchen, die diabetogene Aktivität vom eigentlichen somatotropen Hormon abzutrennen (86, 87); obwohl er verschiedene Verfahren benutzte, war keine wesentliche Änderung im Verhältnis der somatotropen (Wachstums-) Aktivität und der diabetogenen Aktivität bei den gewonnenen Fraktionen zu verzeichnen. Es scheint, daß beide Wirkungen von derselben Struktur des Eiweißmoleküls abhängen.

Von den chromatographischen Verfahren zur Somatotropinreinigung wurden vor allem Säulen mit Hyflo-Super-cel (Kieselgur) verwendet (20). Im sauren pH-Bereich tritt Adsorption des Hormons ein, die Elution wird dann durch Erhöhung des pH-Wertes der Elutionsflüssigkeit vorgenommen, ohne daß jedoch das Hormon inaktiviert wird. Ferner werden Säulen von Carboxymethylcellulose verwendet, an denen das Hormon aus Acetatpuffer vom pH 5,0—5,5 adsorbiert wird. An Säulen von Diäthylaminoäthylcellulose arbeitet man mit der Lösung des Hormons im Boratpuffer pH 8,6; hierbei gelang es jedoch nicht, Somatotropin von Luteotropin abzutrennen. Durch Erhöhung der Molarität des Boratpuffers erzielte man Adsorption von Luteo-

tropin und anderen begleitenden Eiweißkörpern an der Säule, während Somatotropin durchfloß (26). Ansonsten wurden zur Elution verschieden konzentrierte NaCl-Lösungen benützt. Mittels Carboxymethylcellulose gelang die Darstellung eines homogenen Somatotropinpräparates. In einem anderen vorgeschlagenen Verfahren (83) erfolgt die Reinigung an Carboxymethylcellulose mittels essigsaurer Lösung von 0,001 M $CaCl_2$.

In letzter Zeit wurden Somatotropinpräparate auch aus menschlichen Hypophysen, die aus Sektionsmaterial gesammelt wurden, dargestellt (39). Das Präparat wurde dann durch präparative Elektrophorese gereinigt (24) und schließlich in kristallinem Zustand dargestellt (siehe Lewis, U. J., Brink, N. G.: J. Amer. Chem. Soc. 80, 4429, 1958). Außer dem Hormon aus menschlichen Drüsen wurden auch Präparate aus Affendrüsen bereitet und die physikalischen und physikalisch-chemischen Eigenschaften verglichen (68).

Chemische und physikalisch-chemische Eigenschaften des Somatotropins (5, 58, 61, 62). Für das somatotrope Hormon der Rinderdrüsen führt Li die folgenden Konstanten an:

Isoelektrischer Punkt	pH	6,85
Diffusionskonstante	D_{20}	$7{,}15 \cdot 10^{-7}$
Viskositätskoeffizient		7,64
Molmasse		
aus den analytischen Daten . .		43575
aus den osmotischen Messungen .		44250
aus den Viskositätsbestimmungen		29300
aus der Sedimentation		44000

Smith (94) führt an, daß das Hormon in schwachalkalischen Lösungen homogen ist, während es in niedrigerem pH-Bereich Neigung zur Bildung höhermolekularer Aggregate zeigt. Als normal wird die Molekulgröße 40200 angegeben.

Hinsichtlich der chemischen Zusammensetzung des Somatotropins von Rinderhypophysen wurden folgende Angaben ermittelt:

Gehalt an C	46,35%	*Gehalt* an N.	15,50%
H	7,07%	*Amino*-N .	0,76%
S	1,30%	*Amido*-N .	1,20%

Das Hormon enthält weder Phosphor noch eine Zuckerkomponente. Das Verhältnis der sauren und basischen Gruppen im Molekül beträgt 9,8 : 13,4. Die Zusammensetzung der Aminosäuren im Molekül von Somatotropin aus Rinderhypophysen wurde papierchromatographisch mit Hilfe der Dinitrophenylderivate in Hormonhydrolysaten untersucht (67). Für die Molmasse 45757 ergeben sich insgesamt 396 Reste, und zwar:

	R	A	M
Glutaminsäure	50	33	36
Asparaginsäure	35	26	31
Serin	22	20	20
Threonin	26	13	14
Glycin	20	15	14
Alanin	31	11	14
Prolin	14	10	12
Valin	14	9	10
Methionin	7	6	4
Leucin + Isoleucin	76	41	38
Phenylalanin	*27*	*16*	*14*
Tyrosin	11	7	5
Lysin	23	12	12
Histidin	7	5	5
Arginin	26	13	14
Tryptophan	3	1	1
Cystein —	8	8	4
NH_2—	30		

R = Hormon aus Rinderdrüsen, *A* = aus Affendrüsen, *M* = aus menschlichen Drüsen. — Gesamtzahl der Aminosäurenreste bei *A* 241, bei *M* 245 (68).

Auch Somatotropin aus Walhypophysen wurde bereits in sehr reinem Zustand dargestellt (PAPKOFF, H., LI, CH. H.: J. biol. Chem. 231, 367, 1958). Die Molmasse betragt 39000, der isoelektrische Punkt liegt bei p_H 6,2.

Als Endgruppen wurden beim Hormon der Rinderhypophysen *Alanin* und *Phenylalanin* im Verhältnis 1 : 1 festgestellt, woraus sich ergibt, daß das Hormonmolekul offenbar aus zwei Peptidketten besteht, an deren N-Ende sich die beiden erwähnten Aminosäuren befinden (54, 63). REID (87) betonte die Notwendigkeit der Anwesenheit der freien ε-Aminogruppen des Lysins fur die biologische Aktivität des Hormons. *Nach Acetylierung der α-Aminogruppen wird die Hormonaktivitat herabgesetzt,* nach Blockierung der ε-Aminogruppen verschwindet sie jedoch vollig. Außer der Wachstumsaktivität wird auch die diabetogene Wirksamkeit beeintrachtigt.

LI CH. H. mit Mitarbeitern verglich in allerletzter Zeit die Zusammensetzung des aus Rinder-, Affen- und Waldrusen isolierten Hormons, insbesondere hinsichtlich der Aminosäurenfolge der N-endstandigen Peptide (J. biol. Chem. 233, 1140, 1958):

Rinder-Somatotropin		*Alanin*-Phenylalanin-Alanin. .
	und	*Phenylalanin*-Threonin-Alanin. .
Wal-Somatotropin		*Phenylalanin* .
Affen-Somatotropin		*Phenylalanin*
menschliches Somatotropin		*Phenylalanin*-Serin-Threonin . .

Durch Kombination zweier Methoden verfolgte LI mit Mitarbeitern (J. biol. Chem. 233, 1143, 1958) weiter die Aminosaurenfolge der C-terminalen Peptide:

Schaf-Somatotropin	[illegible]
Wal-Somatotropin	
Affen-Somatotropin	
menschliches Somatotropin	*Phenylalanin*-Leucin .

Dabei wurde festgestellt, daß das endstandige Penylalanin fur die biologische Aktivität nicht unbedingt notwendig ist.

Das som [illegible]
gefuhrten [illegible]

Hormons in einer in siedendes Wasser eingetauchten Lösung vollig zerstört, und zwar bei pH 4,0, 7,5 und 8,9. Bis zu 60° C bleiben 0,02 % Hormonlösungen bei pH 7,0 klar, bei 70° tritt jedoch bereits Trubung infolge Denaturierung des Eiweißes ein. Bei 80° C beginnt das Eiweiß auszuflocken. Im allgemeinen verträgt das Hormon insbesondere kein saures Medium und ist hier viel labiler als in mäßig alkalischem Gebiet.

Die Hormonlösung ist bei pH 7,5—8,0 und 5° C relativ bestandig, wie durch Bestimmung der Aktivität des Hormons nach 3jahriger Aufbewahrung festgestellt wurde (34). Nach Acetylierung des Hormons mit Keten, wobei 35 % der Tyrosinhydroxyle und 75 % der Aminogruppen acetyliert wurden, verlor das Hormon die biologische Aktivitat völlig. Ebenfalls durch Einwirkung von salpetriger Säure wird die Wirksamkeit des Hormons gänzlich aufgehoben, so daß die freien Aminogruppen fur die biologische Aktivitat wichtig sind. Nach der Einwirkung von Eisessig auf das Hormon 30 Minuten hindurch bei 70° C wies das ursprünglich homogene Präparat bei der Elektrophorese zwei Komponenten und beträchtlichen Aktivitatsverlust auf (64). Ellis und Mitarbeiter (27) geben die maximale Hormonstabilität im Bereich von pH 5,0—11,5 an.

Neuerdings wurde nachgewiesen (s. Lazo-Wasem, E. A. mit Mitarbeitern: Endocrinology 63, 831, 1958), daß die im sauren und alkalischen Medium dargestellten Hormonpräparate bei verschiedenem pH unterschiedliche Stabilität besitzen. Das im sauren Medium extrahierte Hormon besaß volle Aktivität in klaren Lösungen bei pH 3 und 11, in Suspensionen war es jedoch unwirksam.

Durch Einwirkung von Harnstoff auf 1%ige Somatotropinlösung bei Normaltemperatur änderte sich die Hormonaktivitat bei pH 7,0 (24 Stunden) nicht. Nach der Reaktion des Hormons mit Jod sank die biologische Wirksamkeit, Dejodierung fuhrte wiederum zu Steigerung der Aktivität. In Harnstofflosung konnte ein höherer Jodierungsgrad der Tyrosinreste als bei nativem Hormon erzielt werden (60). Pepsin und Trypsin beeinträchtigen die Wirksamkeit von Losungen des somatotropen Hormons stark Mittels Carboxypeptidase wurden aus dem Somatotropinmolekül zwei Phenylalaninreste abgespalten, ohne daß die Hormonaktivitat grundsätzlich geschadigt wurde (45). Aus Versuchen uber die Einwirkung von Chymotrypsin auf Losungen von reinem Somatotropin stellte Li (66) die Schlußfolgerung auf, daß fur die biologische Aktivität wahrscheinlich nicht das ganze Molekul des nativen Hormons unerlaßlich ist, sondern nur ein Teil

Neuerdings wurden die Eigenschaften des Somatotropins aus Rinderdrusen und aus menschlichen Drusen verglichen; in letzterem Fall wurde festgestellt, daß die Molmasse niedriger ist, offenbar 15—20000 (24) Li mit Mitarbeitern (68) verglich in ahnlicher Weise das Somatotropin der Rinder-, Affen- und menschlichen Drusen. Es wurde festgestellt, daß sich das Somatotropin der menschlichen Drusen und Affendrusen vom Hormon der Rinderdrusen außer durch einen niedrigeren Wert der Mol-

masse (ca. 26000) auch durch die Zusammensetzung unterscheidet (s. S. 478). Die einzige endständige Gruppe scheint hier Phenylalanin zu sein. Die Hormone aus den menschlichen Drüsen und Affendrüsen haben auch einen niedrigeren pH-Wert des isoelektrischen Punktes als das Rindersomatotropin. Diese Untersuchungen sollten die Unterschiede aufzeigen, welche begründen würden, warum das Somatotropin der Rinderdrüsen nach Verabfolgung beim Menschen inaktiv ist. Dies ist eines der seltenen Beispiele für die bisher ermittelte Artspezifität eines Hormons; bekanntlich ist der Großteil der Hormone bei allen höheren Wirbeltieren wirksam.

Biologische Bewertung des Somatotropins. Zur Standardisierung des Hormons wurden mehrere Verfahren vorgeschlagen (4, 11); einmal verfolgen sie die im Organismus nach der Hormonzufuhr ausgelösten morphologischen Veränderungen, zum andern sind es biochemische Verfahren. In die erste Gruppe gehören vor allem Teste, welche die Gewichtszunahme der Versuchstiere nach der Verabreichung des Präparates oder die im Knorpel der Tibien ausgelösten Veränderungen verfolgen. Die biochemischen Verfahren verfolgen die Veränderungen der stickstoffhaltigen Substanzen im Blut, den Phosphorspiegel im Serum, ferner die alkalischen Phosphatasen, die Konzentrationsabnahme von Glutathion in der Leber oder schließlich die Biosynthese der Eiweißkörper mittels markierter Präcursoren. Von den biochemischen Methoden hat sich aber vorläufig keine als Routinemethode der Hormonbewertung eingeführt, und zwar hauptsächlich wegen der geringen Spezifität dieser Verfahren.

Evans und Simpson (29) schlugen im Jahre 1931 einen *Gewichtstest* vor, der an 220—280 g schweren weiblichen Ratten ausgeführt wird, bei denen gerade das Körperwachstum beendet war. Man ermittelt das weitere Fortschreiten des Wachstums nach der Verabfolgung von Somatotropin an Tiergruppen zu je 10 Stück; 20 Tage hindurch werden s.c. oder i.p. Injektionen des Hormonpräparats, insgesamt 17 Dosen, verabreicht. Die Dosierung wird so gewählt, daß in der erwähnten Zeit eine Gewichtszunahme um 40—60 g erzielt wird. Die *Aktivitätseinheit* ist dann jene tägliche Hormongabe, die in 20 Tagen Gewichtszunahme um 40 g bewirkt. Die Verwendung normaler Ratten mit ausgeglichenem Körpergewicht besitzt den Vorteil, daß die Methode für verschiedene Arbeitsstätten verhältnismäßig leicht durchführbar und anspruchslos ist, ein Nachteil ist jedoch die verhältnismäßig geringe Empfindlichkeit des Tests und die Notwendigkeit, das Hormon ziemlich lange verabreichen zu müssen.

Eine anspruchsvollere Methode ist das Gewichtsverfahren, das *hypophysektomierte* weibliche Ratten verwendet (30). Man arbeitet wiederum mit Gruppen von mindestens 10 Versuchstieren, die im Alter von 28—30 Tagen hypophysektomiert wurden. Mit der Darreichung des Hormonpräparates beginnt man den 10. bis 14. Tag nach der Operation, und 9mal innerhalb von 10 Tagen wird eine bestimmte Dosis intraperitoneal verabreicht. Die *Aktivitätseinheit* bei diesem Test ist die tägliche Hormon-

gabe, die nach 10 Tagen einen Gewichtszuwachs von 10 g bewirkt. Zur Erhöhung der Genauigkeit wird das Präparat manchmal 15 Tage hindurch verabfolgt.

Die dritte Methode der Titration des Somatotropins beruht auf der Fähigkeit dieses Hormons, das *Knochenwachstum* zu beeinflussen. Diese Methode beschrieb erstmals EVANS mit Mitarbeiter (31) im Jahre 1943. Als Kriterium der Wirksamkeit des verabreichten Hormons dient hier das Wachstum des proximalen Epiphysenknorpels an der Tibia hypophysektomierter Ratten. 26—28 Tage alte weibliche Ratten werden hypophysektomiert, und 12—13 Tage nach der Operation beginnt man mit der täglichen Verabfolgung intraperitonealer Injektionen, insgesamt 4mal 24 Stunden nach der letzten Injektion erfolgt Autopsie; jedem Tier wird die rechte Tibia entnommen, daraus werden Schnitte angefertigt, die man in Formol fixiert und sodann nach dem Verfahren mit $AgNO_3$ und $Na_2S_2O_3$ bearbeitet Die Breite des nicht calcifizierten Teils der Knochenepiphyse wird unter dem Mikroskop mikrometrisch bestimmt. Diese Methode wird wegen ihrer hohen Empfindlichkeit und Genauigkeit am häufigsten verwendet (44).

Eine weitere Testmethode für Somatotropin ist das Verfahren der Beeinflussung des Körpergewichtes bei der speziellen Rasse der *Zwergmäuse*. Die Empfindlichkeit der Methode ist sehr gut, jedoch bereitet die Züchtung der genannten Rassen von Mäusen Schwierigkeiten. Die einzelnen Methoden wurden von zahlreichen Autoren verglichen (34, 40, 48), sowohl hinsichtlich der Empfindlichkeit und Genauigkeit als auch hinsichtlich der relativ leichten Durchführbarkeit BARTLETT mit Mitarbeiter (14) veröffentlichte ein biochemisches Verfahren der Bewertung von Somatotropinpräparaten bei Hunden, die mit einer Diät von hohem Eiweißgehalt gefüttert wurden. Man verfolgt hier die Stickstoffretention als Kriterium der Hormonaktivität. Zur Bestimmung der *diabetogenen Aktivität* des Hormons auch in Gemischen mit anderen Hormonen wurde eine Methode beschrieben, die die Beeinflussung des Glucosegehaltes bei hyperglykämischen Mäusen mit erblicher Fettsucht verfolgt (70).

Biologische Eigenschaften des Somatotropins. Das Hormon entsteht in den acidophilen Zellen des Hypophysenvorderlappens und wirkt vor allem auf das Gesamtwachstum des Körpers, sowohl des Skeletts als auch der Muskulatur, und zwar bei hypophysektomierten Tieren sowie in einem gewissen Grad auch bei normalen Tieren (2, 3, 8, 9, 10). Durch seine Hypersekretion erklärt sich eine Reihe von Symptomen bei Akromegalie sowie auch bei Gigantismus. Dieser Wirkungsmechanismus ist vorläufig noch nicht ganz geklärt Es wurde die Stimulierung des Thymus in Erwägung gezogen, obwohl man heute den direkten Einfluß des Thymus auf das Wachstum bezweifelt, ferner der Einfluß des Hormons auf die Steigerung der Utilisation hauptsächlich der stickstoffhaltigen Substanzen aus der Nahrung. Es war kein Einfluß auf die Beschleunigung der Ossifikation der Knochen zu beobachten, der Vorgang wird nur bis zum Erreichen des Optimums gefördert. Mit Untersuchungen

über die Ablagerung des radioaktiven Calciumisotopen in den Knochen unter dem Einfluß von Somatotropin beschaftigte sich Ulrich mit Mitarbeitern (106).

Selye (90) stellte bei normalen verschieden ernährten Ratten fest, daß Somatotropin die Verwertung der aufgenommenen Nahrung erhöht, wenn sie ad libitum dargeboten wird; bei beschränkter Nahrungsaufnahme bewirkt es keine Gewichtserhöhung gegenüber den Kontrolltieren. Eine gewisse Resistenz gegenüber dem verabreichten Hormon bei von Versuchsbeginn ad libitum gefutterten Tieren wird nicht durch Antihormonbildung hervorgerufen. Ein hochwirksames Somatotropinpräparat bewirkte weder bei normalen noch bei hypophysektomierten Meerschweinchen Gewichtszunahme des Körpers oder der Organe (74). Das Somatotropin der Rinderdrusen rief erhöhtes Korperwachstum auch bei Fischen — jedoch nur im Fruhjahr — hervor. Bei Wiederholung der Versuche im Sommer konnte diese Wirkung nicht festgestellt werden (102). Bei hypophysektomierten Männchen von Fundulus heteroclitus wurden die Wirkungen von Somatotropinpräparaten aus Fisch- und Rinderdrusen verglichen, wobei die Zunahme des Körpergewichtes und der Korperlänge gemessen wurde. Das Hormon aus Rinderdrusen erwies sich hierbei als wirksamer (79).

Das somatotrope Hormon regt das Wachstum des Foetus bei Ratten nicht an, sondern wies im Gegenteil Hemmwirkung auf, es scheint also, daß das Wachstum des Embryo nicht direkt von der Hypophyse gesteuert wird (72) Bei diesem Hormon wurde ahnlich wie auch bei Cortison Hemmaktivitat beim Wundheilen festgestellt (98). In Gewebskulturen ermittelte man Stimulierung der Zellteilung bei Fibroblasten von Rattenembryen (75); mitogenetischer Effekt desHormons wurde sowohl bei Präparaten der Darmmucose (51) als auch bei Rattenleber (101) gefunden. Interessant sind die Feststellungen über dieBeeinflussung derHamatopoese durch Somatotropin. Bei hypophysektomierten weiblichen Ratten des Stammes Sprague-Dawley wurde Hypoplasie des Knochenmarks festgestellt, nach Verabreichung von Somatotropin gleicht sich das Bild des Knochenmarks sowie das Blutbild aus (71).

Gunstige Ergebnisse mit Somatotropin erzielte man bei der Beeinflussung von experimenteller tuberkulöser Infektion bei Ratten und bei Mausen (53), ferner bei der kombinierten Therapie mit Streptomycin bei tuberkuloser exp. Infektion am Kaninchenauge (19). In dem letztgenannten Fall hatte Somatotropin allein in der Dosis 300 μg/kg keinen besonderen Effekt. Bei exp. Infektion mit *Trypanosoma* [illegible] Cortison [illegible]

[illegible]

gegenuber der Cortisonwirkung sichergestellt wurde (109) Auch gegenuber ACTH weist Somatotropin einen gewissen Antagonismus auf, wie bei Versuchen zur Beeinflussung des Nervensystems festgestellt wurde (52). Somatotropin bewirkt auch pathologische Veränderungen in den Gelenken, die durch Nebennierenrindenhormone beeinflußt werden konnen (88)

Nach langerer Darreichung regt Somatotropin das Auftreten von Geschwülsten bei Ratten an. Es wurde ermittelt, bis zu welchem Ausmaß dieses Hormon das reticuloendotheliale System der Versuchstiere blockieren kann; das Wachstumshormon war jedoch ohne Einfluß auf die phagozytare Aktivitat des RES (95). Eine gewisse Schutzwirkung von Somatotropin wurde bei Rtg-bestrahlten Tieren festgestellt. Das Wachstum der Tiere und die Entwicklung der Organe wurden auf die Norm gebracht, es gelang jedoch nicht, bei den bestrahlten Tieren die Mortalität herabzusetzen (91). Obwohl es in der Literatur Berichte daruber gab, daß Somatotropin nicht als Antigen wirkt, stellte MORRISON mit Mitarbeiter (76) beim Kaninchen die Bildung von Antikörpern gegenuber einigen in verschiedenen Laboratorien bereiteten Somatotropinpräparaten fest.

SEGALOFF mit Mitarbeitern (89) und GEMZELL und Mitarbeiter (38) beschrieben die Bestimmung von Somatotropin im menschlichen Plasma. Die Werte bei Normalen *betrugen bis zu 40 µg/ml Plasma, ein hoher Gehalt wurde bei Akromegalie* festgestellt, bei Panhypopituitarismus fehlte das Hormon im Plasma gänzlich. Nach Markierung des Hormons mit J^{131} wurde die Lokalisierung des Hormons nach der Verabreichung an Ratten verfolgt. Aktivität wurde in Pankreas, Nebennieren, Leber, Milz, Schilddruse, Nieren und Thymus nachgewiesen Im Pankreas überwog die Lokalisierung in den Inseln, jedoch keineswegs in den β-Zellen (97).

Wie aus dem Vorangehenden hervorgeht, ist Somatotropin in seiner Wirkung auf die verschiedensten Gewebe des Organismus ausgerichtet, also nicht auf ein einziges Organ, wie dies bei den ubrigen Hypophysenvorderlappenhormonen der Fall ist. Die Beeinflussung der einzelnen Zellsysteme erfolgt direkt ohne Vermittlung irgendeiner Druse. Es handelt sich also um ein Hormon vom metabolischen Typ (67), das die Biosynthese und Spaltung der drei wichtigsten Gruppen von Nährstoffen, d. h. von Eiweißkörpern, Lipiden und Kohlenhydraten, beeinflußt.

Im *Eiweißstoffwechsel* bei den höheren Wirbeltieren setzt Somatotropin die Menge des durch den Harn ausgeschiedenen Stickstoffs herab, bewirkt Zunahme des Reststickstoffs im Blut und Stickstoffretention im Organismus. Hypophysektomie wirkt bei Tieren gerade umgekehrt. Bei der Untersuchung der Hormonwirkung auf die Verteilung der Eiweißkörper in Geweben bei Ratten wurde ungleiches Wachstum der einzelnen Organe festgestellt (41) In der Steuerung des Stickstoffgleichgewichtes im Organismus spielen allerdings noch verschiedene andere Faktoren und auch das [illegible]

Schlußfolgerung *gelangte man nach Versuchen uber den Einfluß der Kombination* beider Hormone auf den Einbau des mit N^{15} markierten Glycins in das Eiweiß bei Hunden Ferner verfolgte man die Biosynthese von mit S^{35} markierten Albuminen unter *dem Einfluß von Somatotropin und ACTH (107). Das Wachstumshormon*

regt die Biosynthese an, ACTH unterstützt die Spaltung der Albumine, Thyroxin erhöht die Wirkung des Somatotropins nicht. Es konnte auch *die anabolische Wirkung* von Somatotropin bei Versuchen mit Leberperfusion (32) nachgewiesen werden, ferner der Einfluß auf den Gehalt an Eiweißkörpern und Nucleinsäuren in Leberzellen bei *hypophysektomierten Ratten (100)*.

Somatotropin beeinflußt ferner in bedeutsamer Weise den Stoffwechsel der *Lipide* (103). Das Hormon beschleunigt die Mobilisierung des Depotfetts im Organismus und die Ablagerung der Triglyceride in der Leber (42). Man verfolgte auch den Oxydasengehalt und den Einfluß des Hormons auf die Bildung von Acetessigester im Lebergewebe. Nach Applikation von Somatotropin bei normalen Kaninchen treten beträchtliche Veränderungen in der Verteilung der Lipide in Plasma und Leber ein, hier wurde ein Ansteigen der Werte um 84 und 64% festgestellt; in der Aorta hingegen sinken die Werte ungefähr um 25% ab. Das Hormon ruft auch gesteigerte Biosynthese der Phospholipide in Plasma und Leber hervor. Die beschriebenen Wirkungen können durch die primäre adipokinetische Wirkung des somatotropen Hormons erklärt werden (23) Bei der Untersuchung des Gehaltes der Lipoproteine im Serum wurde nach Hormonzufuhr vorübergehende Senkung, später dann ein starker Anstieg festgestellt, insbesondere der Fraktion α_1 mit Albumincharakter (32). Durch *tägliche Hormonzufuhr wurde bei Kühen erhöhte Milchproduktion ausgelöst*, der Fettgehalt in der Milch wurde jedoch nicht beeinflußt (92).

Die diabetogene Wirkung des Hypophysenvorderlappens (12) wurde ursprünglich *einem besonderen Hormon zugeschrieben, eine Reihe von Arbeiten zeigte jedoch* schließlich, daß das somatotrope Hormon auch diese Aktivität besitzt und der diabetogene Faktor der Hypophyse also mit dem Somatotropin identisch ist (86, 87). Reid stellte ferner fest, daß ACTH die diabetogene Wirkung des Somatotropins verstärkt (87) Cotes (22) hält das diabetogene Prinzip der Hypophyse für einen Komplex, der Somatotropin und Corticotropin enthält. Es wurde festgestellt, daß Somatotropin befähigt ist, Hypersensitivität gegenüber Insulin aufzuheben, und man spricht direkt von seiner Antiinsulinwirkung. Das Hormon bewirkt Hemmung der peripheren Utilisierung von Zuckern im Organismus (18). Nach Hypophysektomie wurden Herabsetzung des Blutzuckerspiegels und erhöhte Empfindlichkeit gegenüber Insulin beobachtet *(99), gleichzeitig wurde der Einfluß von Cortison und* Nebennieren untersucht. Bei adrenalektomierten Hunden konnte aufgezeigt werden, daß außer Somatotropin auch Luteotropin diabetogen wirkt, während Corticotropin wirkungslos war (47). Bei Ratten konnte auch durch große Hormondosen keine Glykosurie hervorgerufen werden (69)

Interessant sind ferner die Erkenntnisse über die Wirkung von Somatotropin auf die Pankreasfunktion. Histologisch wurden Veränderungen in der Größe der Zellkerne bei den α-Zellen nach Hypophysektomie festgestellt; Verabfolgung von Somatotropin bewirkt wiederum Normalisierung, und es treten angeblich regressive Ver-

änderungen bei den Kernen der β-Zellen der Pankreasinseln auf (49). Nach Verabfolgung der Suspension aus Schafshypophysen gewonnener acidophiler Granula an Ratten wurde Hypertrophie der α-Zellen der Pankreasinseln hervorgerufen, die mit der Wirkung von gereinigtem Somatotropin identisch war (46). BATTS mit Mitarbeitern (16) stellte bei teilweise pankreatektomierten Ratten nach Somatotropinzufuhr Hypertrophie der β-Zellen der Inseln fest, die dann angeblich auf den erhöhten Blutzuckerspiegel besser ansprechen. Somatotropin soll angeblich auch die Sekretion des Glucagons unterstutzen (36), und außerdem wurde nach Somatotropinzufuhr im Blut ein weiterer hyperglykämisierender Faktor gefunden, der mit Glucagon nicht identisch ist (93).

Bei Versuchen, durch Somatotropin in vitro den Glucoseverbrauch durch ein isoliertes Rattenzwerchfell zu beeinflussen, wurden an verschiedenen Arbeitsstätten vollkommen entgegengesetzte Ergebnisse erzielt. In allerletzter Zeit zeigte sich, daß die Wirkung von der Wahl der verwendeten Pufferlösung stark abhängig ist (84). Durch Somatotropin wurde die endogene Respiration des Uterusgewebes gehemmt, was durch 2,4-Dinitrophenol aufgehoben werden kann. Auch hohe $K^{\cdot}$-Ionen-Konzentration schränkt die hemmende Wirkung des Hormons ein. Das Hormon erhöht im Uterusgewebe die Menge energiereicher Phosphate (37). Enzymologische Messungen zeigten, daß Somatotropin den Spiegel alkalischer Phosphatasen und den Gehalt mineralischen Phosphors im Plasma erhoht (105) Insbesondere wurde auch der Gehalt an verschiedenen Enzymen in der Leber nach der Verabreichung von Somatotropin an Versuchstiere verfolgt. Man stellte erhohten Gehalt an Alanin-Glutamin-Transaminase und D-Aminooxydase fest (17), ferner an der reduzierten Form des Diphosphopyridinnucleotids (DPNH), obwohl das Hormon auf die oxydierte Form (DPN) nur geringen Einfluß besaß (43) Bei der Untersuchung der Ketogenese bei Lebergewebsschnitten hypophysektomierter Ratten stellte man wesentliche Senkung gegenuber den Präparaten normaler Tiere fest. Nach der Verabreichung von Somatotropin an hypophysektomierte Ratten im Laufe von 15 Tagen nach der Operation gleicht sich der ketogene Defekt in Gewebsschnitten aus der Leber dieser Tiere aus. 30 Tage nach der Operation ist Somatotropin auf diesen Effekt bereits ohne Wirkung (104).

Therapeutische Verwendung von Somatotropin. An dieser Stelle wäre es zwar möglich, die einzelnen Versuche zur Verwendung des Hormons in der klinischen Praxis aufzuzahlen, positive Ergebnisse wurden sich darunter doch nur sehr wenige finden, und zwar nicht nur deswegen, weil das reine Hormon verhältnismäßig unzuganglich ist, sondern eher deshalb, daß die klinischen Bemuhungen, das Somatotropin der Rinderhypophysen zur prinzipiellen Indikation *hypophysärer Nanismen* zu verwenden, durchweg negativ verliefen. Man mußte schließlich feststellen, daß diese Praparate bei Menschen unwirksam sind (55, 81). In allerletzter Zeit stellte LI

mit Mitarbeitern (66,$_{1958}$) fest, daß nach Einwirkung von Chymotrypsin auf Somatotropin der Rinderdrüsen durch teilweise Hydrolyse Präparate gewonnen werden, die in ihren physikalisch-chemischen Eigenschaften an das Somatotropin menschlicher Drusen erinnern. Bei der klinischen Untersuchung derartiger Präparate wurde bereits nach der Tagesdosis von 10 mg Hormon Stickstoffretention festgestellt, ohne daß diabetogene Wirksamkeit zu beobachten war. Der Einfluß des Präparates auf das Wachstum und auf unbehandelten Diabetes bedarf noch weiterer Untersuchung.

Somatotropin enthaltende Extrakte des Hypophysenvorderlappens wurden auch zur Behandlung von Hyperinsulinismus untersucht (1). Die Sofortwirkung des Hormons auf den Blutzuckerspiegel war jedoch gering. Gewisse Aufmerksamkeit wurde auch der Beziehung von Somatotropin zum Wachstum von Tumoren zugewandt (13) Auf Grund der vorangehenden Feststellung, daß Somatotropin die Proliferation des fibrösen Gewebes anregt, wurde die Anwendung des Hormons bei schweren Knochenbrüchen geprüft (21). Es wurde auch die Verwendung des Hormons in der Hibernationstherapie erörtert (50). Das Hormon wurde ferner bei der Behandlung von Magengeschwüren und Wundliegen klinisch appliziert (85). Im Sinne der Antagonisierung des corticotropen Hormons wurde auch versucht, Somatotropin bei den durch längere ACTH-Zufuhr hervorgerufenen Psychosen anzuwenden (77).

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21. Adrenocorticotropes Hormon [ACTH]

Die grundlegenden Beobachtungen uber den Einfluß der Hypophyse auf die Nebennierenrindentätigkeit stammen von SMITH (161), der seine Ergebnisse im Jahre 1930 veröffentlichte. Nach Hypophysektomie tritt Atrophie des Rindenteils der Nebennieren ein, durch Implantation von Hypophysen oder durch Injektionen ihrer Extrakte wird Hyperplasie des Gewebes des Rindenteils der Nebenniere ausgelost. Das von dem Hypophysenvorderlappen sezernierte Hormon, das die Tätigkeit der Nebennierenrinde anzuregen vermag, wird als *adrenocorticotropes* Hormon, *Adrenocorticotropin* oder kurz *Corticotropin* bezeichnet

Praktische Bedeutung erlangten die Praparate dieses Hormons nach der Entdeckung der bedeutsamen Aktivität des Cortisons und ahnlicher Corticoide bei rheumatischen Arthritiden. ACTH beeinflußt durch Stimulierung der Nebennierenrinde die Sekretion dieses Typs von Corticoiden und hat also im Organismus mit unbeschädigter Funktion dieses Organs eine ahnliche Wirkung, wie nach Cortisonzufuhr festgestellt worden ist. Dieser praktische Gesichtspunkt fuhrte zur intensiven Erforschung des Corticotropins, sowohl hinsichtlich der Technik seiner rationellen Isolierung, seiner Chemie, als auch hinsichtlich seiner biologischen Wirkungen im Organismus Sehr eingehend wird in letzter Zeit auch die Frage der Steuerung der ACTH-Ausschüttung aus der Hypophyse und der Einfluß des Hypothalamus auf diese Funktion untersucht.

Darstellung der Corticotropinpräparate (2, 3, 8, 11, 13). Zur Isolierung des Hormons verwendete man die Hypophysenvorderlappen verschiedener Tierarten, namentlich Schweinehypophysen, die einen hohen Hormongehalt aufweisen, ferner Schaf-, Rinder-, aber auch Waldrusen (17). COLLIP stellte bereits bei der Darstellung anderer Hypophysenhormone fest, daß die Aktivitat des ACTH in der in 75%igem Aceton oder 70%igem Alkohol loslichen Fraktion enthalten ist. Extraktion mit angesauertem Aceton ist fur die Isolierung von ACTH sehr vorteilhaft, namentlich mit Rucksicht auf die erzielten hohen Ausbeuten und den relativen Reinheitsgrad der Präparate. Alkalische, auch zur Gewinnung anderer Hypophysenhormone angewandte Verfahren sind fur ACTH nicht vorteilhaft, und zwar schon wegen der geringen Stabilitat des Hormons im alkalischen pH-Bereich.

SAYERS und Mitarbeiter (150) veroffentlichten im Jahre 1943 ein Verfahren, bei dem man von ganzen Schweinehypophysen ausgeht, die nach Homogenisieren nach

[illegible] und der Niederschlag wird dann wiederholt mit Wasser eluiert, die Fällung mit Aceton wird wiederholt, und mit Hilfe von Fraktionierung durch allmähliches Erniedrigen des pH-Wertes wird Luteotropin entfernt. Nach Aussalzen mit Ammonsulfat bis zur 0,07 Sättigung wird der Niederschlag entfernt und die Flüssigkeit erneut mit Aceton gefällt, nach der Dialyse wird die Lösung auf pH 4,7 gebracht, [illegible] Hormon, berechnet a[illegible] physenvorderlappenhormonen.

Li mit Mitarbeiter (106) geht wiederum von Acetonextrakten der Schafdrüsen aus; die Fällung erfolgte durch Konzentrationserhöhung des Acetons auf 90%, die Eluierung des Niederschlags wurde mit 0,1 M Na_2HPO_4 vorgenommen und die Fal-[illegible] salzen mit 1,65 M Ammonsulfatlösung und schließlich mit 0,54 M NaCl und 1,36 M NaCl wiederholt. Berechnet auf 1 kg ganzer Drüsen erhält man ungefähr 70 mg sehr reines ACTH.

Fishman (67) beschrieb im Jahre 1947 eine abgeänderte Methode, die höhere Ausbeuten liefert, ca. 1 g ACTH auf 1 kg Drüsen. Das Verfahren wurde dann zur Isolierung des Hormons aus Waldrüsen angewandt (83). Aus 1 kg Waldrüsen, die im einzelnen 15—25 g schwer sind, wurden ungefähr 400 mg Hormon gewonnen, nach einem anderen Verfahren dann ungefähr 0,5—0,9 g (26, 29) neben Luteotropin. Ein anderes Isolierungsverfahren aus Schweinedrüsen geht von mittels Aceton getrockneten Drüsen aus, und zur Extraktion dient Essigsäure (132). Die Reinigung erfolgte durch Fällung sowie durch Adsorption und Verteilung zwischen Lösungsmittel. Zur Isolierung aus Schafdrüsen wurde auch Extraktion mittels Lösungen mit Trichloressigsäure benützt (69), die so erhaltene Peptidfraktion wurde durch Papierchromatographie weiter getrennt.

Smith (162) extrahierte Rinder- oder Schweinedrüsen mit wäßrigem Aceton und kam zu ACTH-Präparaten, die frei von den übrigen Hypophysenvorderlappenhormonen sind. In Schweinedrüsen wurden ungefähr die zehnfache Menge ACTH wie in Rinderdrüsen festgestellt (140). Am vorteilhaftesten scheint das im Jahre 1951 von Astwood und Mitarbeitern (21) veröffentlichte Verfahren zu sein. Die Extraktion wird mit 0,1 N Essigsäure vorgenommen, und die Reinigung erfolgt dann durch Adsorption an Oxycellulose mit nachfolgender Elution mit 0,1 N HCl. Auf diese Weise erreicht man ungefähr 40fache Anreicherung des Hormons gegenüber dem ursprünglichen Extrakt.

Ein vereinfachtes auf derselben Grundlage beruhendes Verfahren teilte ferner Bartholomew (25a) mit.

DIXON (52) erzielte bei Verwendung des Kunstharzes IRC-50 (XE 64) bis 100fache Anreicherung. Die Hormonlösungen waren unbeständig, ließen sich jedoch durch Zusatz von 0,2% Phenol stabilisieren. Die als homogen angesehenen Präparate wurden im Jahre 1951 von G. P. HESS durch Elektrodialyse in eine niedermolekulare wirksame Komponente und eine höhermolekulare unwirksame Komponente aufgetrennt. LI und Mitarbeiter (113) trennten die gereinigten ACTH-Präparate durch die CRAIGsche Verteilungstechnik zwischen 2,4,6-Collidin und Wasser in drei verschiedene Polypeptidkomponenten. LESH und Mitarbeiter (104) trennten die nach Pepsinhydrolyse des gereinigten ACTH-Präparates aus Schweinehypophysen gewonnenen Peptide durch Gegenstromverteilung und isolierten ein 100mal wirksameres Peptid. Aus einem klinischen ACTH-Präparat wurden durch Gegenstromverteilung einige hochwirksame Polypeptidtypen gewonnen, von denen eine Fraktion mit der niedrigsten Molmasse der weiteren Trennung durch 720 Operationsstufen unterworfen wurde; das gereinigte Präparat wurde sodann analysiert (159)

Auch chromatographische Verfahren wurden zur Reinigung von Corticotropinpräparaten häufig verwendet. Insbesondere das erwähnte Astwoodsche Verfahren bewährte sich wegen seiner Selektivität. LI (112) bereitete durch ein kombiniertes Verfahren mittels Aussalzen und Chromatograpie an Oxycellulose ein Corticotropin, das er als Präparat E bezeichnete, und beschrieb seine physikalisch-chemischen und biochemischen Eigenschaften. WHITE (179) benutzte zur Reinigung von ACTH ein Verfahren mit Ionenaustauschern, das derart gewonnene einheitliche Präparat bezeichnete er als *Corticotropin A* und beschrieb dessen Aminosäurenzusammensetzung. Ein chromatographisch homogenes Präparat wurde ferner durch Aussalzen, Adsorption an Oxycellulose und Trennung an Kieselgur erhalten (157): es besaß ungefähr 300 Aktivitätseinheiten in 1 mg. Die ACTH-Konzentrate wurden in Phosohatpuffer an Carboxylkunstharz chromatographiert und die gewonnenen Komponenten als A_1, A_2 und A_3 (51) bezeichnet. Bei Verwendung des Kunstharzes Amberlit IRC-50 (XE-64) bewährte sich ferner zur Chromatographie von ACTH eine Pufferlösung von Pyridin mit Essigsäure und Wasser (52). Ein grundsätzlich neues Reinigungsverfahren des ACTH benützte TAKEDA (168a), und zwar Fällung mit Rivanol.

Die einzelnen Autoren bezeichneten ihre sich abweichend verhaltenden ACTH-Präparate aus Schweinehypophysen auf verschiedene Weise; die Situation wurde bald unübersichtlich und deutete an, daß die ACTH-Aktivität nicht an eine einzige Molekulstruktur gebunden ist. THOMPSON und FISCHER machten den Vorschlag, vier Präparattypen zu unterscheiden, und zwar native nichthydrolysierte oder schwach hydrolysierte Präparate, ferner die Präparate nach Pepsinspaltung des nativen ACTH, dann nach Pepsin- und Säurespaltung und schließlich nach Hydrolyse mittels Säure (171). Dem nativen Typ entspricht *Corticotropin A*, dem gespalteten Typ sodann *Corticotropin B*. Manchmal wurden die Präparate auch mit α und β bezeichnet. Isolierung und Eigenschaften von *α-Corticotropin* aus Schafhypophysen beschrieb LI

mit Mitarbeitern (114). Das Verfahren umfaßt Fraktionierung mit Dioxan, Zonenelektrophorese an Stärke, Chromatographie an Ionenaustauscher und Gegenstromverteilung. *Es existiert angeblich ein labiler Präcursor dieses Corticotropins.*

Zwei Arten wirksamer Polypeptide wurden auch aus Rinderdrüsen isoliert und als A_1 und A_2 bezeichnet. Hierbei wurden niedrigere Ausbeuten ermittelt, und die gewonnenen *Präparate hatten auch niedrigere Aktivität als bei der Isolierung* von ACTH aus Schweinedrusen (179, 1956). Bei Verwendung entfetteter Drüsen und Extraktion mit Methanol und Essigsäure waren die Ausbeuten am günstigsten, *dennoch aber immer niedriger als* bei Schweine- oder Schafdrusen (98). Das Hormon aus Rinder- und Schafdrüsen besitzt identische Aminosäurenzusammensetzung und übereinstimmendes Verhalten bei Chromatographie an der Saule von Amberlit IRC-50, *ebenso auch bei Gegenstromverteilung* (115). *Es wurde auch die Darstellung von* ACTH aus menschlichen Hypophysen beschrieben, und zwar wurden aus 18 Drusen 119 mg Präparat mit der Aktivität von 2,2 I E/mg gewonnen (84)

Vom praktischen Gesichtspunkt wurden drei Arten von Corticotropinen ohne Rucksicht auf die Herkunft unterschieden: *rohes Corticotropin*, das als klinisches Präparat zugänglich ist, ferner *Corticotropin A*, d. h. gereinigtes ungespaltenes ACTH, *und Corticotropin B*, d h. *niedermolekulares* Corticotropin (184). *Es wurden* auch gewisse Unterschiede bei Präparaten aus getrockneten oder frischen Drüsen (64, 82)
[illegible] Prapa-
[illegible] al-
[illegible] nd
Gegenstand zahlreicher Patentanmeldungen, häufig wird hier Reinigung mittels Oxycellulose angewandt. SCHAFER (152) uberprufte die einzelnen Stufen der fabrikmaßigen ACTH-Erzeugung namentlich hinsichtlich der Aktivitatsverluste und beschaftigte sich mit der Ermittlung der Faktoren, welche die Stabilität der Hormonpräparate beeinflussen. Wichtig ist, daß die klinischen Praparate *frei* von *Vasopressin* sind oder zumindest der Gehalt dieses den Blutdruck beeinflussenden Hormons minimal ist (175) PERNIS und Mitarbeiter (133) verglichen die Zusammensetzung einiger Handelspräparate von ACTH. Die Eigenschaften des in der UdSSR hergestellten ACTH beschrieb BROUDE (32).

Vom Gesichtspunkt der Untersuchung der chemischen Struktur des ACTH sind am wichtigsten die Praparate des gespaltenen Hormons, ob nun durch Pepsin oder durch milde saure Hydrolyse. Mit der Analyse derartiger *Polypeptidgemische nach* der Spaltung des nativen ACTH sowie auch mit Versuchen zur Isolierung der corticotrop wirksamen Spaltstücke beschaftigte sich eine Reihe von Autoren (24, 30, 35, 64, 102, 109, 110, 111). Nach der Spaltung von *ACTH mit Pepsin erhielt* man das bereits erwähnte *Corticotropin B*, das aus dem Peptidgemisch des Hydrolysats isoliert wurde und 300mal hohere Wirksamkeit als der ubliche ACTH-Standard besaß (183). *Corticotropin B wird von einer mit dem Kationenaustauscher* Amberlit IRC-50

beschickten Säule festgehalten, während die unwirksamen Komponenten selektiv eluiert werden. Corticotropin B wird dann durch verdünnte Salzsäure in Freiheit gesetzt, aus der Säule herausgelöst und als Hydrochlorid frei von anorganischen Salzen, isoliert (143). Auch nach dem Craig-Verfahren kann Corticotropin B in einheitlicher Form isoliert werden. Es wurde entweder von frischen Schweinehypophysen oder von getrockneten Drusen ausgegangen (64). Praparate von Corticotropin B besaßen auch chromatophorotrope Aktivitat (183).

ACTH-Praparate mit *protrahierter* Wirkung werden durch Kombination des Hormons mit verschiedenen Zusatzen hergestellt, die entweder seine Resorption nach dem Einspritzen verandern oder seine Zersetzung durch Gewebsenzyme verlangsamen. Man verwendet Präparate, die mit Gelatine oder ähnlich wie bei Insulin mit Zink und Protamin (89), mit Zinkphosphat in wäßriger Losung (128), ferner mit Polyphloretinphosphat (86, 176), mit phosphoryliertem Hesperidin (45), mit verschiedenen Wachsen (33), Polyvinylpyrrolidon oder Aluminiumverbindungen (131) dargestellt wurden. Wahrend das native ACTH starke komplexbildende Fahigkeiten besitzt, bilden die hydrolysierten Corticotropinpraparate nur in beschranktem Ausmaß Komplexe (62).

Chemische und physikalisch-chemische Eigenschaften des ACTH (5, 7)
Die in der letzten Zeit fur ACTH benutzten Reinigungsverfahren zeigten, daß die Mehrzahl der fruher beschriebenen Hormonpräparate ein Gemisch von Polypeptiden war, so daß auch die Angaben uber die physikalisch-chemischen Konstanten und gegebenenfalls uber die Zusammensetzung dieser Praparate mit den Konstanten des reinen Hormons nicht gleichzusetzen sind Li (108) gab als Molmasse des ACTH der Schafs- und Schweinedrusen einen Wert von ungefahr 20000 an, fur den isoelektrischen Punkt die Werte 4,70 bzw. 4,80 Neuerdings wird die Molekulgroße auch fur das native Hormon als niedriger angegeben. Fur *β-Corticotropin* ergibt sich nach den Messungen in der Ultrazentrifuge der Wert 4567 (159), der mit dem minimalen aus dem Verhaltnis der enthaltenen Aminosauren berechneten Wert ubereinstimmt. Der isoelektrische Punkt wurde bei pH 4,6, fur *α-Corticotropin* bei pH 6,6 ermittelt (116_{1955})

Das Hormon ist in Wasser und in 60—70 %igem Alkohol leicht loslich. Die Angaben uber seine Fallung mit verschiedenen Reagenzien beziehen sich auf das „native" Hormon mit der hoheren Molmasse. Fast vollstandig kann es aus der Losung von 2,5 %iger Trichloressigsaure oder mittels 20 %iger Sulfosalicylsaure und durch 5 %iges Bleiacetat gefallt werden. Das Hormon ist gegenuber Inaktivierung durch Warme, insbesondere in schwach sauren Losungen, bis zu einem gewissen Grade resistent; im alkalischen Bereich wird jedoch das Hormon rasch zerstort. Li und Pedersen (109) zeigten, daß das Hormon in Gegenwart von Saure ohne Aktivitatsverlust zu kleineren Fragmenten dissoziieren kann.

Wie bereits angedeutet, führt die Spaltung des „nativen" Hormons durch Pepsin nicht zur Herabsetzung der corticotropen Aktivität, bei der Spaltung mit Trypsin bis zu 18% Spaltung ist das Ergebnis ähnlich. Die gewonnenen Peptide besitzen die volle Aktivität des ursprünglichen Hormons. Möglicherweise lagert sich das aktive Peptid insbesondere bei einem pH nahe dem Neutralpunkt mit anderen Peptiden zu Komplexen verschiedener Molmasse zusammen. Das wirksame Peptid wurde als adrenocorticotropes Peptid, ACTP, bezeichnet, und nach der Isolierung und Charakterisierung wurde dieses Polypeptid aus Schweinehypophysen *Corticotropin B* genannt. Mit Pepsin kann es nicht mehr weiter gespalten werden, durch Trypsin oder saure Hydrolyse wird jedoch seine Aktivität aufgehoben.

Durch die Analyse der endständigen Gruppen der isolierten einheitlichen Peptide *Corticotropin A* und *Corticotropin B*, ferner durch Vergleich der Spaltprodukte dieser Polypeptide nach der Einwirkung verschiedener Enzyme und durch Säurehydrolyse wurde allmählich die Reihenfolge der Aminosäuren ermittelt, deren Gehalt zunächst nach der völligen Hydrolyse beider Peptide durch Säuren aufgeklärt worden war (121). Parallel zu der Strukturaufklärung von Corticotropin A und B, ferner β-Corticotropin aus Schweinehypophysen verlief die intensive Erforschung des α-Corticotropins aus Schafshypophysen. Mit der Isolierung und Strukturaufklärung von Corticotropin A beschäftigten sich WHITE und Mitarbeiter, Armour Lab., Chicago, Illinois (179), mit der von Corticotropin B FOLKERS und Mitarbeiter, Merck Lab., Rahway, New Jersey (64), von α-Corticotropin LI und Mitarbeiter, Hormone Research Lab., Univ. California, Berkeley (116), von β-Corticotropin BELL und Mitarbeiter, Amer. Cyanamid Co., Stanford Lab. (159). Die von den einzelnen Arbeitsgruppen ermittelten Ergebnisse stimmen miteinander gut überein.

Als N-terminale Aminosäure bei allen aktiven Corticotropinpräparaten wurde *Serin* ermittelt (66), an das weiter Tyrosin gebunden ist. Die C-terminale Aminosäure bei Corticotropin A, α und β ist *Phenylalanin*, das wiederum an den Rest der Glutaminsäure gebunden ist und diese weiter an Leucin. Das komplette Polypeptid der angeführten drei Corticotropine besteht aus 39 Resten von 15 verschiedenen Aminosäuren. *Corticotropin B entsteht durch Abtrennen von 11 Aminosäureresten vom Carbo-*

Ser—	Tyr—	Ser—	Met—	Glu—	His—	Phe—	Arg—	Try—	Gly—	Lys—	Pro—	Val—	Gly—	Lys—	Lys—
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

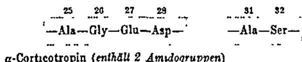
													NH_2		
Arg—	Arg—	Pro—	Val—	Lys—	Val—	Tyr—	Pro—	Asp—	Gly—	Ala—	Glu—	Asp—	Glu—	Leu—	Ala—
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32

Glu—	Ala—	Phe—	Pro—	Leu—	Glu—	Phe
33	34	35	36	37	38	39

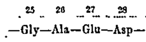
β-Corticotropin (*enthält 1 Amidogruppe*)

xylende des Polypeptids von Corticotropin A, so daß es insgesamt 28 Aminosäurereste enthält. Die C-terminale Aminosäure ist die Asparaginsäure.

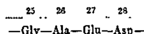
Dies ist die komplette Aminosäurenfolge bei β-Corticotropin. Ferner sind die strukturellen Unterschiede der übrigen als Individuen isolierten Corticotropine angeführt:



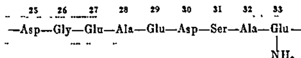
α-Corticotropin (*enthält 2 Amidogruppen*)



Corticotropin A (*enthält keine Amidogruppen*)



Corticotropin *B* (*enthält keine Amidogruppen*)



Corticotropin der Rinderdrüsen* (*enthält 1 Amidogruppe*)

Wie daraus ersichtlich ist, unterscheiden sich Corticotropin A, α und β durch die Folge der Aminosäuren 25—28, außerdem durch die Sequenz der Aminosäurereste in Stellung 31 und 32. Während bei Corticotropin A keine Amidogruppen festgestellt wurden, wurden für Corticotropin α zwei Amidogruppen gefunden, bei Corticotropin β eine Amidogruppe, und zwar die Glutaminsäure in Stellung 30. Der Unterschied in der Zahl der Amidogruppen beim Corticotropin α und β, die aus Schweinehypophysen dargestellt wurden, kann durch die verschiedenen Bedingungen bei der Darstellung beider Präparate erklärt werden. Allerdings kann die abweichende Aminosäurenfolge in Stellung 25—28 nicht auf diese Weise gedeutet werden.

Bell und Mitarbeiter (159) isolierten nach der Spaltung des β-Corticotropins mittels Pepsin 3 einheitliche aus 28, 30 und 31 Aminosäureresten bestehende aktive Polypeptide, die jedoch nicht die Aktivität des ursprünglichen Corticotropins erreichten. Ferner wurde festgestellt, daß der Hauptteil der Ketten mit 24 Aminosäureresten für die Aktivität am wichtigsten ist. *Corticotropin B* enthält bezüglich der qualitativen Zusammensetzung gegenüber *Corticotropin A* kein Leucin im Molekül.

Die Richtigkeit der Identifikation einiger durch Spaltung des *Corticotropins A* gewonnenen Peptide wurde durch die Synthese dieser Peptide bestätigt. So wurden

* s. Li Ch. H. und Mitarbeiter: J. amer chem. Soc 80, 2587, 1958.

drei Dipeptide dargestellt: *Glutamylphenylalanin, Seryltyrosin* und *Arginyltryptophan* (180), ferner ein Pentapeptid: *Ser-Tyr-Ser-Met-Glu* (87) und schließlich *Phenylalanyl-Arginin* und *Glutamyl-histidyl-phenylalanyl-arginin* (87). Wie im Kapitel uber das Melanophorenhormon gezeigt werden wird (s. S. 510), hängt ein Teil der Struktur des Corticotropins ganz parallel mit der Struktur dieses Hypophysenzwischenlappenhormons zusammen. Daher wurde auch bei ACTH-Präparaten melanophore Aktivitat gefunden.

Nach Einwirkung von *Aminopeptidase auf Corticotropin A wurde festgestellt*, daß das N-terminale Serin fur die Aktivität unerläßlich ist (179). Beim Studium der Bedingungen der Stabilität der ACTH-Präparate zeigte sich (72), daß das Hormon insbesondere in neutralen wäßrigen Lösungen sehr unbeständig ist, Säurezusatz wirkt angeblich stabilisierend. Dennoch kommen die Präparate zumeist in lyophilisierter Form in den Handel. Nach der Einwirkung von Oxydationsmitteln verliert das Hormon seine Aktivität, in dieser Richtung wurden insbesondere der Einfluß von *Wasserstoffperoxyd und Perjodat uberpruft (50). Durch Reduktion gelingt es manchmal, die* Hormonaktivitat wiederherzustellen, der Einfluß von Perjodat und Peroxyd ist jedoch sehr verschieden. Durch Einwirkung von KBH_4 konnten weder die corticotrope noch die melanophore Aktivitat wiederhergestellt werden. Bei Inaktivierung mittels Peroxyd bei pH 7—7,5 gelingt die Reaktivierung mit SH-Substanzen bei niedrigerem pH in der Warme (48). Da das Hormon kein Cystein enthält, ist nicht klar, welche Gruppen an den Oxydoreduktionsvorgangen eigentlich beteiligt sind.

Durch Acetylierung mit Keten geht die ACTH-Aktivität allmahlich verloren, durch Einwirkung von salpetriger Saure wird die Wirksamkeit sehr rasch zerstort, auch Formaldehyd bewirkt Inaktivierung des Hormons. Geschwind und Li (71) *beschaftigten sich in letzter Zeit mit der Einfuhrung von Guanidin in α-Corticotropin.* Durch Jodierung wird das Hormon inaktiviert, es sind daher die freien Tyrosinreste fur die Aktivitat wichtig. Bei der Untersuchung des empfindlichen Volumens des ACTH, das im trockenen Zustand mit beschleunigten Deuteronen beschossen wurde, wurde ein dem Gewicht von 2400 ± 800 entsprechender Wert ermittelt (41).

Bei Handelspraparaten von ACTH fand man bei der Kontrolle der deklarierten Wirksamkeit von Praparaten verschiedener Firmen ein gewisses Absinken der Aktivität, *nach weiteren 2 Jahren Lagerzeit konnte jedoch kein weiteres Absinken festgestellt* werden (77, 78).

Biologische Bewertung des ACTH-Präparate (4, 15). Fur die biologische Titration dieses Hormons werden direkte oder indirekte Methoden meist an Ratten, und zwar bei hypophysektomierten oder normalen, angewandt. Die direkten Methoden beruhen auf dem Studium des Einflusses des Hormons auf die Nebennieren, und zwar entweder durch den *Gewichtstest oder durch die Bestimmung* des *Ascorbinsäure*gehaltes in den Nebennieren nach der Verabreichung von ACTH-Praparaten oder durch

Beeinflussung der Sekretion der Rindenhormone bei Nebennieren in vitro. Bei den indirekten Methoden werden die im Organismus nach ACTH-Zufuhr ausgelösten Veränderungen verfolgt, die durch erhohte Corticoidausschuttung vermittelt werden.

Collip und Mitarbeiter (46) arbeiteten bereits im Jahre 1933 ein Verfahren aus, bei welchem man das Gewicht einer exstirpierten Nebenniere bei hypophysektomierten Ratten mit dem Gewicht einer zweiten Druse nach Verabfolgung eines ACTH-Präparates vergleicht. Hypertrophie der Nebennieren bei den Versuchstieren wird von zahlreichen Faktoren beeinflußt, und zur quantitativen Bewertung von ACTH-Praparaten bewährten sich am besten hypophysektomierte Tiere (124). Mannlichen Ratten wird im Alter von 40 Tagen die Hypophyse entnommen; unmittelbar nach der Operation wird ihnen das zu untersuchende Hormonpräparat täglich i.p. oder s.c. injiziert. Das Nebennierengewicht bei den operierten Kontrolltieren sinkt von ursprünglich 36 mg auf ca. 12 mg ab; die Hormonmenge, welche das Nebennierengewicht bei dem ursprunglichen Wert erhält, wird als eine *Erhaltungseinheit* bezeichnet (160). Li und Evans empfahlen, das Gewicht der Nebennieren auf 100 g Korpergewicht der Versuchstiere auszudrucken. Das ACTH-Präparat wird 15 Tage hindurch verabreicht (42). Das Verfahren des Wagens der Nebennieren wurde auch bei infantilen Mausen, Ratten oder Huhnern angewandt.

Reiss (139) verfolgte die histologischen Veranderungen, die nach der Hormonzufuhr bei hypophysektomierten Ratten in den Nebennieren auftraten. Nach Hypophysektomie beobachtet man insbesondere Schwinden der Lipoide in dem Rindenteil der Druse: die minimale Hormonmenge, die den deutlichen Beginn der Redistribution der Lipoide in der Nebennierenrinde auslöst, wird als die *Einheit* bei diesem Test betrachtet. Li und Sayers verwandten das Verfahren beim Testen gereinigter ACTH-Praparate und halten die Methode fur sehr empfindlich.

Im Laufe der Zeit wurde zur ACTH-Bestimmung zumeist die von Sayers und Mitarbeitern (151, 177) im Jahre 1948 ausgearbeitete Methode verwendet, die den Ascorbinsauregehalt in den Nebennieren verfolgt. Hierbei werden männliche Ratten im Gewicht von 120—160 g verwendet, bei denen ungefahr 24 Stunden nach der Hypophysektomie die linke Nebenniere in Pentobarbitalanasthesie exstirpiert wird. Die Losung des zu untersuchenden Hormonpraparates wird intravenos verabfolgt und nach einer Stunde wird der Ascorbinsauregehalt in der verbliebenen Nebenniere bestimmt, und die ermittelte Abnahme des Gehaltes beim Vergleich mit dem Gehalt in der linken Nebenniere ist das Maß der ACTH-Aktivitat. Das Verfahren wurde vielfach modifiziert, es wurden dekapitierte Tiere verwendet, die durch kunstliche Atmung weiter am Leben erhalten wurden (76), Versuchstiere mit durchtrenntem Ruckenmark (178), infantile Tiere (55), normale Ratten nach der Injektion von Cortexon (85) oder nach Zufuhr von p-Oxypropiophenon (129), ferner wurde das Präparat bei hypophysektomierten Ratten subkutan verabreicht (142). Der letztgenannte Test wird in der USP XV empfohlen. 0,5 μg reines ACTH-Praparat auf je 100 g

Körpergewicht setzt den Ascorbinsäuregehalt in den Nebennieren um ungefähr 60 mg%, berechnet auf die frische Drüse, herab.

Die Bestimmung der ACTH-Aktivität *in vitro* beruht auf der Stimulierung der Corticoidbiogenese in der Nebennierenrinde nach Einwirkung dieses Hormons bei der Bebrütung von Nebennierengewebe in Gegenwart von Sauerstoff (148). Der Vorzug des Verfahrens ist, daß es an einem Tag durchgeführt werden kann; der Effekt ist spezifisch für ACTH, andere Hypophysenhormone stören hierbei nicht. Der Test ist größenordnungsmäßig ungefähr so empfindlich wie die Methode mit der Ascorbinsäure. Man verwendet isolierte Rattennebennieren, die Ergebnisse werden z. B. nach der Steigerung der Corticoidbiogenese nach Umrechnen auf Cortison ausgewertet. Auf diese Weise wurden ACTH-Präparate von verschiedenem Reinheitsgrad bestimmt, und die Ergebnisse stimmten mit den Messungen nach dem Verfahren nach SAYERS überein (119). Ein anderes Verfahren zur Bewertung des ACTH beruht auf der Messung der Ausscheidung der 17-Oxysteroide im venösen Blut der Nebennieren bei Hunden nach Zufuhr des corticotropen Hormonpräparates (127).

Zu den indirekten Methoden der ACTH-Bestimmung gehört der *Eosinophilentest*, der ebenfalls bei Menschen erprobt wurde (164, 172), ferner das Verfahren, nach welchem die *Thymusrückbildung* bei Ratten nach ACTH-Zufuhr verfolgt wird (61). Ein kombiniertes Verfahren der Bewertung der ACTH-Präparate führte HOHLWEG mit Mitarbeitern ein (88); hierbei werden gleichzeitig das Gewicht der Nebennieren und das Thymusgewicht bei intakten Ratten gemessen. Der Wert des Koeffizienten $K = 10\,N/T$ (N Nebennierengewicht, T Thymusgewicht) ist ein Maß der Aktivität des Präparats; bei den Kontrolltieren beträgt er ungefähr 2, bei maximaler Reaktion ungefähr 8. Normale Präparate werden 3 Tage hindurch verabreicht, protrahierte einmalig, und am 4. Tag wird die Wirkung gemessen. Man arbeitet mit relativ großen Tiergruppen mit doppelter Kontrolle ohne Hormon und mit dem Standardpräparat.

Es wurde versucht, die *chromatophore* Aktivität des ACTH bei Amphibien auch zur Bewertung von Corticotropinpräparaten zu verwerten (54, 165, 170). Zumeist wurden hierzu Laubfrösche verwendet, GESCHWIND und Mitarbeiter (69) zeigten jedoch, daß bei den einzelnen Präparaten keine Parallelität zwischen der corticotropen und melanophoren Aktivität besteht. Man bediente sich auch der mikroskopischen Methode der Bewertung von ACTH-Präparaten an Froschhaut; diese Methode beruht auf demselben Prinzip, ist jedoch zur Routinekontrolle der ACTH-Aktivität wenig geeignet (96).

PARROT (130) bestimmte nach dem Gewichtstest und nach dem Test nach SAYERS die ACTH-Aktivität im Blutplasma. Bei Normalen wurden 50—100 μg% Hormon gefunden, bei Patienten mit CUSHINGschem Syndrom 100—200 μg%. Im Blut der normalen Ratte wurde die ACTH-Aktivität nach Parabiose mit einer hypophysektomierten Ratte bestimmt (31).

Zur quantitativen Bewertung der Corticotropine sind die biologischen Methoden unerläßlich. Man versuchte eine Reihe von Präparaten verschiedener Herkunft mittels

Papierelektrophorese zu bewerten und stellte bei pH 5,5 und 8,6 3—4 Fraktionen fest; die Präparate wurden auch polarographisch untersucht. Diese physikalisch-chemischen Teste gestatten jedoch nur eine qualitative Untersuchung der verschiedenen Corticotropinpräparate (68). Die biologischen Teste nebeneinander wurden von REISS mit Mitarbeiter kritisch verglichen (141) und drei Verfahren als verlaßlich bezeichnet: die histologische Methode, das Verfahren nach SAYERS und die Beobachtung der Stoffwechselsteigerung in Nebennierenschnitten mittels radioaktivem Isotopen P^{32}. Die Gewichtsmethode hält er fur weniger verläßlich. Ähnlich halt auch PARKES (129) die Teste durch Messung der erhohten Widerstandsfähigkeit gegenuber Anoxie nach ACTH-Zufuhr fur wenig spezifisch. Er verglich auch die Moglichkeiten, die Bestimmungen an nicht hypophysektomierten Tieren vorzunehmen. Zur richtigen Bewertung der Handelspraparate von ACTH mussen noch Proben auf die Aktivität anderer eventuell vorhandener Hypophysenhormone durchgefuhrt werden (60, 138), besonders, wie bereits erwähnt, auf den *Gehalt an Vasopressin.* Gewisse Diskrepanzen zwischen der biologischen Aktivitat der Praparate und ihrer klinischen Wirkung werden neuerdings durch die Unterschiede in der Art der Applikation erklart (65). Das Verhältnis der biologischen Aktivitat bei i.v. und s.c. Verabfolgung im biologischen Test ist angeblich ein maßgebender Index der klinischen Wirksamkeit (65).

Die *internationale Einheit* der Corticotropinwirksamkeit ist die Aktivitat von 1 mg Standardpräparat *Armour La-1-A 1944*, welches den internationalen Standard darstellt.

Biologische Eigenschaften des ACTH (6, 14, 16). Das Hormon wird von den *basophilen* Zellen des Hypophysenvorderlappens sezerniert Im anteromedialen Teil wurde bei Schweine- und Rinderdrusen 4—13mal soviel ACTH wie in den ubrigen Teilen des Vorderlappens festgestellt. Der Hinterlappen enthalt nur geringe Mengen des Hormons (145) Die im Vorderlappen der Schweinedrusen festgestellte Gesamtmenge betragt 0,2—0,4 I. E. ACTH-Aktivitat, berechnet auf 1 mg Trockengewicht. Eine ähnliche Menge wurde auch in den Drusen bei Menschen und Walen aufgefunden.

In den letzten Jahren wurde der Steuerung der Sekretion dieses Hormons große Aufmerksamkeit zugewandt (9, 120, 123, 149, 153). Man stellte fest, daß durch Cortisonzufuhr der ACTH-Gehalt in den Hypophysen bei Hunden herabgesetzt wird (59); die nach operativer Entfernung einer Nebenniere ausgeloste erhohte ACTH-Ausschuttung läßt sich auch durch Zufuhr von Cortisol oder Cortexon unterdrucken (101) Der Corticoidgehalt des Blutes beeinflußt daher die Sezernierung des ACTH. Außerdem wurde deutlicher Einfluß des Zentralnervensystems sichergestellt. Nach Unterbrechung der Verbindung zwischen dem Hypothalamus und der Hypophyse nahm die ACTH-Sekretion ab, als jedoch die Versuchstiere der Kalte ausgesetzt wurden, trat erhohte ACTH-Ausschüttung ohne direkte Verbindung der Gehirnzentren mit der Hypophyse ein (25)

Auch der Hypophysenhinterlappen spielt für die ACTH-Sekretion eine gewisse Rolle (120, 149); es wurde die Aktivität des Vasopressins in Erwägung gezogen, die die ACTH-Ausschüttung anregt. Der Faktor hängt bis zu einem gewissen Grade mit der antidiuretischen Aktivität der Hypophysenhinterlappenaktivität zusammen. Neuerdings wird als stimulierende Substanz ein von Vasopressin unterschiedliches niedermolekulares Peptid angesehen (75), das jedoch auch in Handelspräparaten von Vasopressin mitenthalten ist, ähnlich wie es die aus Pferdedärmen isolierte Substanz P begleitet (167). *Der Stimulierungsfaktor wurde als P_1 bezeichnet. Offenbar sind* an der Steuerung der ACTH-Ausschüttung auch nervöse und humorale Einflüsse beteiligt (19).

Corticotropinaktivität wurde auch in der Placenta (20, 166), ferner im *Blut* (20) und schließlich auch im Harn festgestellt (28, 117, 147, 182). VAN DYKE (173) verfolgte die Zeit, während der das ACTH im Körper wirksam ist, und ermittelte die verhältnismäßig kurze Lebensdauer der Hormonmoleküle von 17 Minuten, für Somatotropin hingegen ungefähr 9 Stunden, und dies bei physiologischem Spiegel beider Hormone bei Ratten. Bei Brütung von ACTH mit dem Blut epinephrektomierter Ratten in vitro wurde kein wesentlicher Einfluß auf die Aktivität des zugeführten Hormons festgestellt (168). Nach s.c. Verabfolgung von 10 USP-E ACTH an hypophysektomierte Ratten wurde bereits nach einer Minute das Hormon im Blut festgestellt; die höchste Konzentration wurde nach 4—8 Minuten erreicht, nach 80 Min. konnte das Hormon bereits nicht mehr im Blut nachgewiesen werden (103). Als mittels J^{131} markiertes Corticotropin im Organismus verfolgt wurde, konnte festgestellt werden, daß es in der Nebennierenrinde und in der Schilddrüse rasch aufgefangen wird (163); die höchste Konzentration war in den Nieren lokalisiert (38), und zwar im *Verhältnis zu der Abnahme im Blut. Das Hormon wird aus dem Organismus eher* auf metabolischem Wege als durch Ausscheidung entfernt (38).

Ein praktisch sehr bedeutsames Problem ist die Art, auf die das Hormon im Körper inaktiviert wird. GESCHWIND und LI verfolgten die Inaktivierung von ACTH durch Gewebsschnitte und Homogenate in vitro und stellten dabei fest, daß die niedermolekularen Präparate rascher inaktiviert werden als die Eiweißpräparate (70). Komponenten, die das Corticotropin im Organismus inaktivieren, sind nach PINCUS (134) das thermolabile Blutprotein und ein in zahlreichen Geweben anwesender Cofaktor. Das Fibrinolysin des Rinderbluts spaltet das Molekül des Corticotropins A in Stellung 8 und 15, was zu völligem Verlust der Hormonaktivität führt. WHITE und GROSS betrachten diesen Mechanismus als Ursache der Inaktivierung des Hormons im Blut (181). Die Leberprotease spaltet das Hormon in Stellung 31 und 35 ohne Verlust der biologischen Corticotropinaktivität. Bei der Darstellung von Corticotropinpräparaten mit protrahierter Wirksamkeit muß also die Aktivität der Enzyme nach Hormonzufuhr im Organismus eingeschränkt werden, vielleicht durch Bindung verschiedener Metallionen an das Hormonpräparat (36). Eine andere Möglichkeit

ist die Einschrankung der Hormonresorption durch Zusatz eines geeigneten Vehikels infolge Hemmung der Gewebshyaluronidase (44).

Die Hauptwirkung des ACTH ist die Anregung der Sekretion der Nebennierenrinde (16). Die einzelnen Rindenzonen bei normalen und hypophysektomierten Tieren, die einer Belastung ausgesetzt waren oder denen man Corticotropin verabreicht hatte, wurden histologisch untersucht (74). Die Zona glomerulosa weist eine gewisse Unabhangigkeit von der Hypophyse auf, dennoch wird ihre Abscheidungstätigkeit ebenfalls von Corticotropin gesteuert. Der Einfluß von ACTH auf die Sezernierung der Rindenhormone wurde in vivo durch die Analyse der in das Blut ausgeschütteten (127, 135, 137) oder durch den Harn ausgeschiedenen Corticoide (40, 122) verfolgt, sowie in vitro bei der Beeinflussung der Biogenese der Corticoide durch Nebennierenhomogenate (81) oder bei Versuchen mit perfundierten Nebennieren der Versuchstiere (81). Sowohl Hypertrophie der Nebennieren, verändertes histologisches Bild der Rinde als auch Anregung der Biosynthese und Ausschuttung der Corticoide dienen als Maß der Aktivitat bei der quantitativen Bewertung der Corticotropinpraparate, wie bereits im vorangehenden Abschnitt erwahnt wurde. Wahrend ACTH in vivo den Ascorbinsäurespiegel in den Nebennieren beeinflußt, war bei Versuchen in vitro mit Gewebe von Schweinedrusen keine Senkung des Vitamin-C-Gehaltes nach ACTH-Einwirkung zu beobachten (93).

Nach Verabreichung von ACTH gemeinsam mit Ascorbinsaure wurde die Wirkung des Hormons bei normalen Ratten nicht beeinflußt, bei hypophysektomierten Ratten wurde sie jedoch verstärkt (53); ähnlich stellte man Aktivitätssteigerung bei Goldhamstern fest, bei denen der Ascorbinsauregehalt in den Nebennieren nach ACTH-Zufuhr nicht absinkt (154). Beim Auflösen des Hormons gemeinsam mit Ascorbinsäure ermittelte Holzbauer mit Mitarbeiter (90) verminderte Hormonaktivität, wogegen Dedman und Mitarbeiter (49) erhöhte Wirksamkeit einer derartigen Lösung feststellten, was sie durch die Schutzwirkung des Vitamins gegenüber der Autooxydation von ACTH erklären.

Den Mechanismus der Stimulierung der Corticoidsynthese unter dem Einfluß von ACTH untersuchten in allerletzter Zeit Haynes R. C und Berchet, L. (*J. biol. Chem.* **225**, 115, 1957; **233**, 1220, 1958) an Homogenaten von Rindernebennieren. Es konnte ermittelt werden, daß das Hormon die Aktivitat der die Umwandlung von Glykogen in Glucose-1-phosphat katalysierenden Phosphorylase steigert; Glucose-1-phosphat wird weiter zu Glucose-6-phosphat isomerisiert und dieses sodann dehydriert. Bei diesem Prozeß wird die reduzierte Form des Triphosphopyridinnucleotids TPNH wiederhergestellt, die zur Biosynthese der Corticoide erforderlich ist.

Die „extraadrenalen" Wirkungen des Corticotropins wurden von Selye (155) behandelt; eine Reihe dieser Effekte hangt sekundär von der Nebennierenrindenfunktion ab. Es handelt sich z. B. um Stimulierung der Thymusinvolution oder um die eosinophile Reaktion; beide indirekten Wirkungen des ACTH dienen, wie bereits erwahnt,

auch zur *Bewertung der Corticotropinpräparate.* ACTH dämpft ähnlich wie Cortison die Entzündungsreaktion der Gewebe (34), ohne jedoch die Ursache der Entzündungen zu beeinflussen. Diese Wirkung wird wahrscheinlich durch verminderte Permeabilität der Kapillaren und der serösen Membranen infolge Einwirkung von ACTH bzw. Cortison ausgelöst. Corticotropin hemmt Wachstum und Entwicklung der Ovarien und des Uterus, ohne jedoch die Sekretion der Gonadotropine durch die Hypophyse zu beeinflussen; das Gesamtwachstum des Organismus ist verlangsamt (58). ACTH antagonisiert den stimulierenden Einfluß des Somatotropins auf die Milchproduktion *(158) bei den Versuchstieren. Die Antiheparinaktivität einiger ACTH-Präparate hängt* wahrscheinlich von einem der in solchen Präparaten enthaltenen Begleitstoffe ab, der keinen Eiweißcharakter besitzt (99). Die Melanophorenaktivität des ACTH bei Amphibien ist durch die Identität eines Teils der Polypeptidkette bei ACTH und dem Melanophorenhormon gegeben (s. S. 510), beide Hormone sind jedoch *nicht identisch.* Das Melanophorenhormon ist corticotrop unwirksam.

Bei gereinigten ACTH-Präparaten wurde starke *adipokinetische* Wirksamkeit beobachtet; nach Hormonzufuhr steigt der Lipoidgehalt in der Leber stark an (107, 146). *Es wurde ein gewisser Synergismus zwischen ACTH und Somatotropin hinsichtlich* der *diabetogenen* Aktivität festgestellt (58, 174) Nach Insulinhypoglykämie steigt der Gehalt des endogenen ACTH im Organismus an (97). In allerletzter Zeit untersuchte Engel mit Mitarbeitern (57) den Einfluß des gereinigten *ACTH* auf den *Glucosestoffwechsel* bei Ratten. Bei hungernden Ratten bewirkt das an Oxycellulose gereinigte Präparat signifikante Ketonämie, die durch i v. Glucosezufuhr nicht beeinflußt wird. ACTH erhöht die *Toleranz* hungernder Ratten gegenüber i.v. eingespritzter Glucose. Größere ACTH-Gaben schützen intakte und adrenalektomierte bei *Normaldiät* gehaltene oder hungernde Ratten vor Hypoglykämie oder durch Insulin ausgelösten Konvulsionen, obwohl paradoxerweise das ACTH-Präparat an sich den Blutzucker (22) unter gewissen Bedingungen herabsetzt. Es wurde auch der *Einfluß von ACTH auf die Eiweißkörper des Blutserums untersucht* (27, 63).

Die antidiuretische Wirkung einiger ACTH-Präparate läßt sich offenbar von den im Präparat vorhandenen Ballaststoffen ableiten, die auch Hypophysenhinterlappenhormone enthalten (92). Nach ACTH-Zufuhr wurde ähnlich wie nach Cortisonzufuhr (73) erhöhte Magensekretion von Säure und Pepsin beobachtet.

Ähnlich wie bei den übrigen Proteohormonen wurden auch bei verschiedenen ACTH-Präparaten die *antigenen* Eigenschaften geprüft (37, 43, 80, 105, 126). Anscheinend werden nicht Antikörper mit Antihormonaktivität gebildet, sondern eher nur Antikörper gegenüber den wirkungslosen Begleitstoffen. Ähnlich wie Cortison senkt auch ACTH die Resistenz des Organismus gegenüber Infektion (39, 94, 144, 156). Bei chronischer s.c. Verabreichung einer größeren Hormonmenge täglich an normale *Ratten wurden Glykosurie und Acetonurie beobachtet,* und es traten umfassende pathologische Veränderungen in Nieren und Herzmuskel auf; ein Teil der Versuchs-

tiere hatte Geschwulste in der Pylorusgegend, und sechs Tiere gingen an schweren diffusen Infektionen zugrunde. Gewichtsverlust und negative Stickstoffbilanz waren bei den Tieren nur vorübergehender Natur (91). Die gleich gefutterte Kontrollgruppe, der nur physiologische Lösung dargeboten wurde, überlebte ohne sichtliche Veränderungen, in der Versuchsgruppe gingen innerhalb 21 Tagen 60% der Tiere zugrunde.

Therapeutische Verwendung des ACTH. Über dieses Problem liegt heute bereits eine ausgedehnte Literatur vor, denn ACTH wurde ähnlich wie Cortison in den letzten Jahren auch klinisch intensiv bearbeitet.

Das Hauptanwendungsgebiet ist die *rheumatische Arthritis*, die auf die klassische Therapie nicht anspricht; hierbei wurden ebenso gute Ergebnisse wie mit Cortison und seinen Derivaten erzielt. ACTH mobilisiert die funktionsfähige Nebennierenrinde und erhöht die Sekretion der Corticoide vom Cortisontyp Ähnlich wie Cortison hat aber auch ACTH keine anhaltende therapeutische Wirkung und ruft eine Reihe unangenehmer Nebenreaktionen hervor. Die Größe der Dosen und die Anwendungsdauer sind sehr individuell. Normale Präparate werden in Intervallen von 6 Stunden intramuskulär verabfolgt oder intravenös durch langsame Infusion der Lösungen zur Anwendung gebracht. Die Präparate kommen lyophilisiert in den Handel, da die Lösungen unbeständig sind. Protrahierte Präparate liegen in Form von Dispersionen vor.

Ein weiteres Anwendungsgebiet sind verschiedene akute *allergische Zustände* bei exfoliativer Dermatitis, Status asthmaticus, ferner *Augenentzündungen* u. ä. m. Verabreichung von Corticotropinpräparaten ist kontraindiziert bei Tuberkulose, dekompensierten Herzfehlern, bei fortgeschrittener Arteriosklerose, Hochdruck, bei Nierenkrankheiten, Zuckerkrankheit, Osteoporosen, bei Magenhypersekretion, in den Frühstadien der Schwangerschaft sowie bei Patienten nach Operationen. Die Anwendung von ACTH ist natürlich auch bei Hypersekretion der Nebennierenrinde, bei CUSHINGschem Syndrom kontraindiziert. Die Erfahrungen mit der klinischen Verabfolgung des Hormons bei einer ganzen Reihe von Krankheiten finden wir in zusammenfassenden Arbeiten, von denen nur einige angeführt werden können (1, 10, 12, 23, 79, 95, 100, 125, 136, 169). ACTH wurde mit Erfolg auch in der Tierheilkunde verwendet (18); hier ist es jedoch ein recht kostspieliges Heilmittel

Corticotropinpräparate sind auch für die *Diagnostik* von Bedeutung, und zwar für die Ermittlung des Funktionszustandes der Nebennierenrinde im Thorn-Test (176)

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22. Melanophorenhormon [Intermedin]

Das Hormon, das die Melanophoren in der Haut von Kaltblütern anregt, wurde im mittleren Teil der Hypophyse aufgefunden. Es ist beim Farbwechsel der Haut dieser Lebewesen wirksam, findet sich jedoch auch bei Warmblütern und bei Menschen. SMITH (38) und unabhängig ALLEN (4) beobachteten bereits im Jahre 1916, daß Frösche nach Hypophysektomie Albinismus aufweisen. Drei Jahre später beschrieb ATWELL (6) Versuche an Kaulquappen, die in verdünnten Lösungen gezüchtet wurden, die den Extrakt aus dem Mittellappen der Rinderhypophyse enthielten. Diese Tiere besaßen eine dunklere Hautfarbe als die normalen Kontrollen der Kaulquappen.

Implantationsversuche fuhrte ALLEN (5) im Jahre 1920 durch. HOGBEN und WINTON (20) beschäftigten sich mit Extrakten des Hypophysenmittellappens und ihrem *Einfluß auf die Dilatation der Melanophoren. Das für diese Wirkung verantwortliche* Hormon wurde als melanophores oder chromatophores Hormon bezeichnet, nach dem Entstehungsort auch als Intermedin oder neuerdings als melanocytenstimulierendes Hormon (MSH). Die Bedeutung dieses Faktors fur Warmblüter, bei denen sich die Hautfarbe nicht ändert, ist bisher nicht ganz klar. Vor kurzem wurde festgestellt, daß das Hormon gewissermaßen dem ACTH nahesteht; die nach der Pepsinspaltung des Corticotropins isolierten Peptide zeigten melanophore Aktivitat (25). In jungster Zeit wurde das Hormon *rein isoliert und chemisch als ein Peptid* mit 18 Aminosaureresten (β-MSH) oder 13 Aminosaureresten (α-MSH) völlig charakterisiert.

Darstellung des Melanophorenhormons. ZONDEK und KROHN (45) gewannen im Jahre 1932 die ersten von Oxytocin und Vasopressin freien Extrakte, welche die Ausbreitung der Melanophoren beeinflußten, und nannten das Präparat Intermedin. Aus den gemeinsam mit dem Hypophysenmittellappen abgetrennten Hinterlappen wurde mittels Aceton Wasser entzogen und das Pulver durch Kochen mit 0,25 %iger Essigsäure 10 Minuten hindurch extrahiert. Nach dem Einengen des Extraktes zur Trockne wurde mit absol. Alkohol wiederholt eluiert. Nach dem Verdampfen wurde der Ruckstand in Wasser gelost und die Losung nach dem Filtrieren zur Zerstörung *von Oxytocin und Vasopressin mit* 1 % NaOH versetzt. Nach Neutralisieren wurde mittels Äther, Aceton und Äthylacetat weiter gereinigt.

STEHLE (39) fällte die mittels Essigsäure gewonnenen Hypophysenhinterlappenextrakte mit dem 5—10fachen Volumen Alkohol, verdampfte die Losung zur Trockne,

extrahierte den Rückstand mit Methanol und fällte nach dem Abtrennen des Rückstands mit Äthylacetat. Nach wiederholter Reinigung gelangte man zu einem Präparat, das beim Test der Ausdehnung der Froschmelanophoren 25mal wirksamer als das Standardpräparat war und nur eine geringe Menge Oxytocin und Vasopressin enthielt.

LERNER und LEE (31) führten im Jahre 1955 ein Isolationsverfahren für das Melanophorenhormon an, bei dem sie von getrockneten Hypophysenhinterlappen ausgingen. Das Acetontrockenpulver wurde mit essigsaurehaltigem Aceton bei 50° C extrahiert, sodann die Fällung durch Steigerung der Acetonkonzentration vorgenommen und die erhaltene Fraktion sodann durch Aussalzen mit NaCl gereinigt. Ferner wurde Reinigung mittels Oxycellulose in essigsaurer Lösung angewandt. Das derart erhaltene Polypeptidpräparat wurde der Gegenstromverteilung unterworfen und war 500mal wirksamer als das Ausgangsmaterial. Später isolierten die genannten Autoren zwei Hormone mit melanocytenstimulierender Wirksamkeit (29). Die Präparate waren frei von pressorischer und ACTH-Wirksamkeit. Unabhängig davon wurde die Existenz von zwei melanophoren Hormonen auch bei Hundehypophysen festgestellt (43). Neuerdings wurden Präparate von Melanophorenhormon der Zonenelektrophorese unterworfen (34) und dabei ein Polypeptid mit niedrigerer Molmasse gewonnen.

Eine weitere Reinigung des Hormons wurde durch die Craig-Technik nach der Isolierung aus Schweine- und Rinderdrüsen verwirklicht (8). Man stellte einige Unterschiede in der chemischen Zusammensetzung der aus den Drüsen beider Tierarten gewonnenen Präparate fest. Zur Trennung verwendete man 0,5%ige Trichloressigsäure und sek. Butanol und erzielte gute Abtrennung von ACTH (7). GESCHWIND und LI (16) extrahierten das Hormon aus Schweinedrüsen mit Eisessig in der Wärme und nahmen die Reinigung sodann durch Fällung mit Aceton und Äther vor. Nach Durchfluß durch eine Oxycellulosesäule wurde weitere Reinigung des Hormons durch Zonenelektrophorese an Stärke und durch Gegenstromverteilung nach CRAIG vorgenommen.

Chemische und physikalisch-chemische Eigenschaften. Die erstmals beschriebenen Eigenschaften des Hormons wurden bei unzulänglich reinen Präparaten ermittelt. Es wurde angeführt, daß das Hormon wasserlöslich, thermostabil und verdünnten Säuren gegenüber relativ beständig ist; durch Einwirkung stärkerer Säuren tritt Zersetzung ein. Auch wurde die Lichtempfindlichkeit des Hormons erwähnt. STEHLE führt in Arbeiten aus dem Jahre 1944 und 1945 (40) für das mittels Pikrinsäure gereinigte Präparat eine 73mal höhere Wirksamkeit als beim Standard und den Gehalt an einigen Aminosäuren an. CHEN und GEILING untersuchten die Wirkung von Alkalien auf das Hormon; in Übereinstimmung mit LINDGREBE und WARING teilen sie mit, daß durch Einwirkung von Alkalien die Aktivität des Hormonpräparates

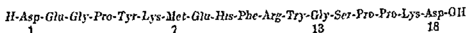
erhöht werden kann. WERLE und SCHÄFER (44) untersuchten den Einfluß verschiedener Fermente auf die Wirksamkeit des Melanophorenhormons.

Eine gewisse Ähnlichkeit des Melanophorenhormons mit dem nach Pepsinvorverdauung von ACTH gewonnenen Peptid stellten JOHNSON und HOGBERG (25) fest. Demnach besitzen ACTH-Präparate eine gewisse Melanophorenwirksamkeit, hingegen besitzt jedoch das reine Melanophorenhormon keine ACTH-Aktivität. Beide Hormone sind also sowohl in biologischer als auch in chemischer Hinsicht voneinander verschiedene Prinzipe (41).

Die Diskrepanz in den Ergebnissen verschiedener Autoren hinsichtlich der Eigenschaften und insbesondere der Molekülgröße des Hormons erklären LEE und LERNER (29) damit, daß einige Autoren mit dem Präparat des α-Melanophorenhormons (α-MSH) und andere mit β-Melanophorenhormon (β-MSH) arbeiteten. Neuerdings stellten die beiden erwähnten Autoren fest (*J. biol. Chem.* 233, 917, 1958), daß aus dem pulverisierten Handelspräparat der Schweinehypophysenhinterlappen durch ein Fraktionierungsverfahren sowohl α-MSH als auch β-MSH dargestellt werden können.

Ein Isolationsverfahren für β-MSH und gleichzeitig auch verschiedene seiner Eigenschaften geben GESCHWIND und LI (16) an. Auf Grund der Beweglichkeit bei der Elektrophorese wurde der *isoelektrische Punkt* dieses Polypeptids bei pH 5,8 ermittelt, bei Berechnung des isoionischen Punktes wurde der Wert 5,9 erhalten. Der isoelektrische Punkt für α-MSH wird bei pH 10,5 und 11,0 angegeben. Als minimale *Molmasse* des β-MSH berechnete man aus dem Aminosäurengehalt den Wert 2177. GINSBURG und Mitarbeiter ermittelten auf Grund von Messungen in der Ultrazentrifuge den Wert 2900. Alle diese Werte gelten für das aus *Schweinedrüsen* isolierte Hormon.

Durch systematische Analyse der Peptide nach enzymatischer Spaltung des Hormons, verbunden mit Aufklärung der Endgruppen, gelang es schließlich, die Reihenfolge der Aminosäuren in dem Hormon mit 18 Aminosäureresten zu bestimmen (β-MSH):

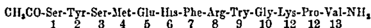


Die im Rahmen angegebene Aminosäurensequenz stimmt auch mit der Reihenfolge der Aminosäuren im Corticotropin überein. Sieben Aminosäuren besitzen also im Molekül beider Hormone dieselbe Reihenfolge, die Aminosäurensequenz bei MSH in Stellung 7—13 entspricht der *Aminosäurenfolge bei Corticotropin in Stellung* 4—10. Diese Ergebnisse für die Hormone aus Schweinehypophysen veröffentlichten unabhängig voneinander GESCHWIND und LI (16) und auch HARRIS und ROSS (18).

Bei der analogen Untersuchung der Zusammensetzung von β-MSH aus *Rinderhypophysen* stellten GESCHWIND und LI (17) fest, daß dieses Hormon den isoelek-

trischen Punkt bei pH 7,0 besitzt und auch aus 18 Aminosäuren zusammengesetzt ist. Die gesamte Aminosäurensequenz stimmt überein, nur in Stellung 2 befindet sich an Stelle der Glutaminsäure des Hormons aus Schweinedrüsen *Serin* beim Hormon aus Rinderdrüsen. Die Änderung in der Gesamtionenverteilung in der Polypeptidkette kommt durch die Verschiebung des pH-Wertes seines isoelektrischen Punktes zum Ausdruck.

HARRIS J. I. (*Biochem.* J. **71**, 451, 1959) führt in jüngster Zeit die Struktur des aus Schweinehypophysen isolierten α-MSH an:



Die Aminosäurenfolge dieses N-Acetyl-tridecapeptid-amids stimmt mit dem N-endständigen Rest des Tridecapeptids des Corticotropins überein (s. S. 494).

Vor kurzer Zeit wurden bereits (HOFMANN, K. mit Mitarbeitern: J. amer. chem. Soc **80**, 6458, 1958) synthetisch einige Peptide der erwähnten Aminosäurenfolge dargestellt, wie sie im Molekül des Melanophorenhormons α-MSH vorkommt; bei einigen wurde in biologischen Testen Melanophorenaktivität festgestellt. Das Pentapeptid *His. Phe. Arg. Try. Gly* besaß die Aktivität $1{,}5 \cdot 10^4$ E/g, *Carbobenzoxy-Ser. Tyr. Ser. Meth. Glu. His. Phe. Arg. Try Gly* 0,8 10^8 E/g.

Biologische Bewertung des Melanophorenhormons. Der grundlegende Test ist hier die Messung der *Ausdehnung* der *Melanophoren* oder allgemein der *Hautpigmentierung* bei Kaltblütern (1, 2, 3). Da verschiedene Faktoren, wie Temperatur, Luftfeuchtigkeit, Licht usw. die Reaktion der Melanophoren beeinflussen, müssen bei den Testen die Standardbedingungen mit Leichtigkeit eingehalten werden können. Als Versuchstiere werden zumeist Vertreter von *Rana temporaria* oder andere Froscharten verwendet. Gewisse Vorteile besitzt auch die Verwendung von *Xenopus* Arten. Es können intakte sowie hypophysektomierte Tiere verwendet werden.

Die Bestimmung der Expansion der Melanophoren erfolgt entweder unmittelbar an lebenden Tieren oder an perfundierten decerebrierten Individuen und schließlich an in Salzlösung suspendierten Hautpräparaten. Oft bedient man sich bei der Messung der HILLschen photoelektrischen Methode mit automatischer Registrierung. Neuerdings wurde ein Verfahren zur Bestimmung des Hormons an *Rana pipiens* beschrieben; die Tiere sind 30—50 g schwer und werden in einer 20—25° C warmen Lösung gehalten, zu der das zu prüfende Hormonpräparat zugegeben wird. Das Dunkeln der Haut wird photoelektrisch gemessen (36).

Derartige objektive quantitative Methoden werden allgemein zur Bestimmung des Melanophorenhormons verwendet. Die Ergebnisse wurden zur Ermittlung der

Genauigkeit des Verfahrens statistisch verarbeitet. Bei Verwendung von ACTH-Präparaten mit Melanophorenaktivität konnte die lineare Beziehung der Gaben zu der ermittelten Aktivität im Bereich von 0,064—6,4 μg auf je einen Frosch ermittelt werden (11). In allerletzter Zeit wurden die einzelnen Verfahren *zur Auswertung* des Melanophorenhormons namentlich hinsichtlich ihrer Genauigkeit von DEUTSCH und Mitarbeitern (12) verglichen.

Die reinsten Präparate des Melanophorenhormons, die GESCHWIND und LI dargestellt hatten, wurden zunächst an hypophysektomierten Fröschen *Rana pipiens nach der Methode der Untersuchung des Melanophorenindex* nach HOGBEN und SLOME(21) titriert; später wurde die objektivere Methode in vitro an Froschhaut angewandt (35), und die nach beiden Methoden gewonnenen Ergebnisse stimmten gut miteinander überein. Die Titration an albinischen Kaulquappen *Hyla regilla* (13), denen das Hormonpräparat injiziert wird, ermöglicht noch Mengen an β-MSH von der Größenordnung $10^{-6}\mu$g nachzuweisen. Das reinste Hormonpräparat aus Schweinedrüsen besaß die Wirksamkeit von $0{,}5 \cdot 10^{7}$ E/mg. Ein IE entspricht ungefähr 10^{4} dieser angewandten Einheiten.

Als *internationale Einheit* der Melanophorenhormonwirksamkeit schlugen LANDGREBE und WARING (28) die Wirksamkeit von 0,5 mg *internationalem Standardpräparat* getrockneter Rinderhypophysenhinterlappen vor. Das erste und zweite internationale Standardpräparat haben hinsichtlich der Melanophorenwirksamkeit beinahe übereinstimmende Aktivität.

Biologische Eigenschaften des Melanophorenhormons. Das Hormon ist vorwiegend in Hypophysenmittellappen, aber auch im Hinterlappen enthalten, über sein Vorkommen im Vorderlappen liegen verschiedene Angaben vor. Die hier ermittelte Melanophorenaktivität kann vom Corticotropin herrühren, das, wie bereits erwähnt, ähnliche chemische Struktur besitzt, und verschiedene ACTH-Präparate weisen wirklich Melanophorenwirksamkeit auf. Die Verschiedenheit beider Faktoren wird jedoch heute nicht mehr bestritten (10, 14, 35, 41, 42).

Durch zytochemische Untersuchung des Hypophysenzwischenlappens beim Frosch *Rana pipiens* wurden zwei Typen intracellulärer Granula festgestellt. Durch Injektion des Melanophorenhormons wurden die Zellen der Pars intermedia nicht beeinflußt, nur die Färbbarkeit mit Bromphenolblau war verändert, ebenso war Thyreoidektomie ohne Einfluß auf diese Zellen. Im Hypophysenmittellappen ermittelte man insgesamt 0,1 μg Hormon (33) Die Melanophorenhormon-Aktivität wurde auch bei menschlichen Hypophysen untersucht (23), Melanophorenwirksamkeit wurde in der Placenta neben ACTH-Wirksamkeit (24) und auch im Blutplasma festgestellt (26).

Es wurde die Aufgabe des Melanophorenhormons bei der Melaninbildung untersuch [illegible]

des [illegible]

Intermedin die Melaninsynthese nicht unmittelbar fordert (27). Ferner verfolgte man die Melanogenese in der Meerschweinchenhaut in den sogenannten *Dendritenzellen*, ihre Beeinflussung durch das Melanophorenhormon, und zwar auch zwecks Ausarbeitung einer Titrationsmethode (37). Inwieweit die Melaninbildung in den Haaren und Federn mit der Wirkung dieses Hormons zusammenhangt, konnte vorlaufig nicht eindeutig entschieden werden. Hinsichtlich der Hautpigmentierung beim Menschen konnte nicht ermittelt werden, daß das Melanophorenhormon bei Negern in einer größeren Menge als bei Weißen gebildet wurde. Anzeichen dafür, daß das Hormon mit der Hautpigmentierung zusammenhangen kann, ist aus der erfolgreichen Beeinflussung von *Vitiligo* zu ersehen; diese Hautkrankheit ist durch pigmentlose Flecke gekennzeichnet (*Leukopathie*).

Die etwaige Mitwirkung des Hormons bei Addisonscher Krankheit und bei Melanosen wurde an zahlreichen Fällen untersucht (32); bei der erstgenannten Krankheit ermittelte man Mitwirkung der Hypophyse bei der pathologischen Pigmentierung, im anderen Fall jedoch nicht. Hogberg beschäftigte sich im Jahre 1953 mit der Frage der Pigmentierung bei Addisonikern und stellte erhohte Intermedinbildung fest; ähnlich waren erhöhte Werte des Melanophorenhormons auch in der Schwangerschaft (19) zu verzeichnen.

Die Untersuchung des MSH-Gehaltes kann beim Studium des Stoffwechsels von Adrenalin und Arterenol, bei malignen Melanomen u. ä. m. (30) von Bedeutung sein. Die Hormone der Nebennierenrinde und des Nebennierenmarks dampfen angeblich die Hormonaktivitat, auch Serotonin wirkt hemmend, Ergotamin und Dibenamin heben im Gegenteil die Hemmung auf. Neuerdings ergab sich, daß das Melanophorenhormon Anomalien in Respirationsversuchen bewirkt, die mit Gewebsschnitten der Brustdruse bei Ratten nach Prolactinzufuhr durchgefuhrt wurden; Prolactin kommt angeblich immer mit MSH gemeinsam vor (9). Ähnlich stellte man Verunreinigungen mit MSH auch bei Praparaten anderer Hypophysenhormone fest. Durch Zusatz von Ascorbinsaure zu Losungen des Melanophorenhormons wird starke Inaktivierung des Hormons ausgelost, wie Versuche ergaben, bei denen zum Schutz der Lösungen vor Oxydation das Vitamin zugesetzt wurde (22).

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23. Oxytocin und Vasopressin

Bereits im Jahre 1895 wiesen OLIVER und SCHÄFER (85) nach, daß nach intravenöser Injektion von Hypophysenextrakt bei Versuchstieren Blutdrucksteigerung eintritt. HOWELL (66) konnte später den Nachweis erbringen, daß der Hypophysenhinterlappen die wirksame Substanz enthält. Im Laufe der Zeit wurden sodann die einzelnen Wirkungen der aus den Hinterlappen dieser Drüsen bereiteten Extrakte beobachtet, insbesondere hinsichtlich der Wirkung auf die Uteruskontraktionen oder allgemein auf die glatte Muskulatur und auch auf die Diurese. CYON (36) führte im Jahre 1898 Versuche zur Ermittlung des chemischen Charakters des Hypophysenhinterlappenhormons aus, das für die Blutdrucksteigerung verantwortlich ist; zu dieser Zeit waren noch keine anderen Wirkungen der Extrakte bekannt.

Es wurde festgestellt, daß die pressorische Wirkung des Hinterlappenextraktes von Blutdrucksenkung abgelöst werden kann, wenn man weitere Injektionen dieses Materials anwendete. Aus dem Infundibulum der Hypophyse dargestellte Alkoholextrakte besaßen nur depressorische Wirkung, und eine Zeitlang wurde daher ein weiteres Hormon vorausgesetzt. Später zeigte sich, daß die Extrakte Histamin enthielten, das für diese Wirkung verantwortlich ist.

Die Arbeiten zur Isolierung des die Uteruskontraktionen stimulierenden Hormons schritten nur langsam vorwärts und dauerten ungefähr 40 Jahre. Gesamtpräparate, welche die pressorische sowie die zweite Uteruskontraktionen auslösende Komponente enthielten, wurden als *Pituitrin* bezeichnet und verhältnismäßig bald in die medizinische Praxis eingeführt. Die pressorische Komponente war hier unerwünscht, und die Forschung war dahin gerichtet, beide Aktivitäten voneinander abzutrennen, was schließlich auch gelang; die pressorische Komponente wurde *Vasopressin* oder *Pitressin* genannt, die auf die glatte Muskulatur wirkende Komponente *Oxytocin* (aus dem griech. οξυς heftig, τοκος Geburt) oder *Pitocin*.

DU VIGNEAUD isolierte schließlich beide Hormone im Reinzustand, bestimmte ihre chemische Struktur und führte mit seinen Mitarbeitern auch die Synthese beider Hormone durch. Es ist dies vorläufig der einzige Fall in der Gruppe der Proteohormone, wo es gelang, die bisher aus Drüsenmaterial isolierten Hormone künstlich herzustellen. Die beiden Hormone Oxytocin und Vasopressin sind gegenüber den übrigen Proteohormonen verhältnismäßig einfache Peptide, sie bestehen aus acht Aminosäureresten. DU VIGNEAUD wurde für diese Arbeiten mit dem Nobelpreis ausgezeichnet.

Darstellung der Präparate des Oxytocins und Vasopressins. Die ersten aktiven Präparate aus Hypophysenhinterlappenextrakten bereitete HOUSSAY im Jahre 1911, und zwar durch Fällung der wäßrigen Extrakte mit basischem Bleiacetat, ferner durch Extraktion mit verschiedenen Lösungsmitteln; er erhielt so ein gelbliches Pulver, das die volle Wirksamkeit einfacher Hinterlappenextrakte besaß. Im Jahre 1912 gewannen ENGLAND und KUTSCHER eine Lösung mit Oxytocinaktivitat, gaben jedoch keine näheren Einzelheiten über deren Darstellung an. FÜHNER (49) untersuchte ein Jahr später eine Reihe von aus Kalbshypophysen dargestellten Präparaten, bei deren Darstellung er Fällung mit Phosphorwolframsäure benützte. Er gewann angeblich schwach gelb gefärbte Kristalle und nannte die Substanz *Hypophysin*. Es handelte sich offenbar um eine nicht identifizierte kristalline Substanz, an die etwas von den Hormonen adsorbiert worden war, ähnlich auch bei den weiteren Fraktionen, die derselbe Autor noch isolierte.

FÜHNER stellte erstmals fest, daß die pressorische und oxytoxische Wirksamkeit nicht an eine einzige Substanz gebunden sind, und dieser Befund wurde später von zahlreichen anderen Forschern bestätigt. ABEL und ROUILLIER (12) beschrieben eine Darstellung der Präparate, wobei von tiefgefrorenen Hypophysenhinterlappen ausgegangen und zur Extraktion 0,35%ige Salzsaure verwendet wurde; sodann wurde der Extrakt mit verschiedenen Fallungsmitteln fur Eiweißkorper gefallt, und es konnten wirksame Präparate gewonnen werden. Wie sich jedoch später zeigte, war der Großteil der Fällungen nicht fur die Hormone, sondern fur verschiedene Ballasteiweißstoffe spezifisch, an welche die Hormone nur adsorbiert waren.

Erst im Jahre 1928 beschrieb KAMM mit Mitarbeitern (71) die Trennung des Vasopressins von Oxytocin. Er ging hierbei von Acetontrockenpulver der Hinterlappen aus, das mit 0,25%iger Essigsaure bei 95° 30 Minuten hindurch extrahiert wurde Nach dem Einengen wurden die Extrakte mit Ammonsulfat ausgesalzen und der gewonnene Niederschlag mit Eisessig extrahiert; der Extrakt wurde hierauf mit Äther und Petroläther gefällt. Aus 100 g Ausgangsmaterial wurden derart 5—10 g Rohprodukt gewonnen. Die Trennung beider Hormonfaktoren erfolgte nach Extraktion mit 98%iger Essigsäure bei 40° C durch Fällung mit dem 2,5fachen Volumen Äther, und zwar wiederholt und durch weitere Fraktionierung. Das von den Autoren als *β-Hypophamin* bezeichnete Vasopressin enthielt noch 3—4% Oxytocin und die als *α-Hypophamin* bezeichnete Oxytocinfraktion noch ungefähr 1—2% Vasopressin.

Im Jahre 1933 veröffentlichten DU VIGNEAUD und Mitarbeiter (128) ein Verfahren, das zu Präparaten von höherem Reinheitsgrad fuhrt, und gaben hierbei einige chemische Eigenschaften der Hormone an, namentlich den Gehalt an Schwefel, Cystin, und Tyrosin. STEHLE (106) führte eine Methode zur Trennung beider Hormone nach der Fällung der Eiweißkorper aus dem Extrakt durch wiederholte Fallung mit Äthylacetat an. Vasopressin bezeichnete er als *Postglobin-V* und Oxytocin als *Postglobin-O*. Zur Grundlage einer Reihe von Verfahren zur Trennung beider Hormone wurde die

Tatsache, daß Oxytocin in verschiedenen organischen Lösungsmitteln löslicher als Vasopressin ist.

Weitere Methoden zur Trennung beider Hormonfaktoren beruhen auf ihrer unterschiedlichen Adsorption an verschiedenes Material, insbesondere wurde Bentonit als Adsorbens verwendet. POTTS und GALLAGHER (90) fraktionierten die Hormone an Permutiten. Vasopressin wurde stärker gebunden als Oxytocin. Beide Prinzipe zur Trennung des Oxytocins vom Vasopressin, ob nun auf Grund der verschiedenen Löslichkeit in organischen Lösungsmitteln oder auf Grund der Adsorption wurden bei der fabrikmäßigen Herstellung von Oxytocinkonzentraten verwendet, und diese Verfahren sind durch zumeist jedoch bereits abgelaufene Patentanmeldungen geschützt. Eine Zeitlang bereitete man nur teilweise gereinigte Hypophysenhinterlappenextrakte, die als Präparate unter der Bezeichnung *Pituitrin* in den Handel kamen. Bei der Herstellung ist es wichtig, die bakterielle Kontamination, die beim Drüsenmaterial bereits am Schlachthof auftritt, einzuschränken (104). Bakterielle Decarboxylasen können Steigerung des Histamingehaltes im Präparat bewirken.

IRVING und Mitarbeiter (67) benutzten zur Trennung von Oxytocin und Vasopressin das Prinzip der unterschiedlichen Beweglichkeit beider Komponenten bei der Elektrophorese. Vasopressin wanderte unter den angeführten Bedingungen 6mal rascher zur Kathode als Oxytocin. Die durch Elektrophorese getrennten Präparate wurden nach Vorreinigung der Extrakte durch Aussalzen mit Ammonsulfat durch Papierchromatographie weiter gereinigt (55).

Mittels der Gegenstromverteilung nach CRAIG gelang es, beide Hormonkomponenten aus vorgereinigten Hypophysenhinterlappenextrakten rein und mit hoher biologischer Aktivität zu isolieren. Diese Arbeiten führte DU VIGNEAUD mit einer Reihe von Mitarbeitern des Dept. der Biochemischen Abteilung des Cornell Univ. Med. Coll. in New York durch (122). Als Ausgangsmaterial diente einmal das Handelspräparat der Firma Parke Davis Pitocin, zum andern Rinderhypophysenhinterlappen. Von den Lösungsmittelsystemen bewahrte sich am besten das Gemisch von 2-Butanol und 0,05% Essigsäure, ferner auch 2-Butanol und 0,01 M NH_4OH. Im erstgenannten System ist der Verteilungskoeffizient für Oxytocin 0,4, im zweiten dann 1,8. Das hierbei gewonnene *Oxytocinpräparat* besaß über 800 Wirkungseinheiten in 1 mg. Aus homogenem amorphem Oxytocinpräparat wurde dann kristallines Flavianat vom Smp. 190—200° C *in Form von Nadelchen dargestellt.*

Zur Gewinnung von *Vasopressin* nach dem Craig-Verfahren wurde das Lösungsmittelsystem n-Butanol und 0,09 M p-Toluolsulfonsäure verwendet. Vasopressin der Rinderdrüsen besaß in diesem System den Verteilungskoeffizienten 1,25, das Präparat aus Schweinedrüsen 0,66. Die Aktivität des Präparates aus Rinderdrüsen betrug 400—500 IE auf 1 mg, bei Vasopressin aus Schweinedrüsen wurde niedrigere Wirksamkeit festgestellt. Neben der vasopressorischen Aktivität wurde auch die antidiuretische Wirksamkeit dieser reinen Präparate untersucht; hierfür ergaben sich

bezüglich der Einheiten dieselben Werte, woraus geschlossen werden kann, daß beide Aktivitäten einer Substanz angehören. Bei reinen Vasopressinpräparaten wurde eine gewisse schwache Wirksamkeit gegenüber der glatten Muskulatur festgestellt, die für Oxytocin charakteristisch ist. Auf 100 Einheiten der pressorischen Aktivität wurden ungefähr 5 IE oxytocischer Wirksamkeit gefunden; dies spricht nicht für eine Verunreinigung des Vasopressinpräparates, sondern ist eine Struktureigenschaft des Vasopressinmoleküls. *Wie im weiteren gezeigt werden wird, ist die Struktur beider* Hormone ziemlich ähnlich.

Beide Hormone können schließlich noch durch ein weiteres Verfahren getrennt werden, *das auf der Adsorption an Silikagel und auf selektiver Elution beruht.* Die Adsorption erfolgt aus 0,3%iger essigsaurer Lösung an besonders vorbereitetem Silikagel. In der Kälte werden beide Hormone adsorbiert. *Oxytocin* wird dann mit der *heißen Lösung von 0,5%iger Essigsäure selektiv eluiert. Zur Gewinnung von Vasopressin* wird die Adsorbenssäule mit der Lösung von wasserfreiem Ammonsulfat und einem Zusatz von Trimethylcetylammoniumbromid in Eisessig gewaschen (siehe FROMAGEOT, P. und Mitarbeiter: *Biochem. Biophys. Acta* **11**, 252, 1953; **12**, 424, 1953); die Adsorption des Hormons wird an Salicylsäure vorgenommen. Nach Auflösen der Säure in Äther wird das Gemisch mit Wasser extrahiert, und nach Lyophilisieren wird das Präparat der *Gegenstromverteilung unter Benutzung des Systems* Wasser-n-Butanol mit Salicylsäure unterworfen

Heute wird neben den aus Drüsenmaterial isolierten Präparaten Oxytocin bereits auch *synthetisch* dargestellt. Diese Präparate enthalten aber nicht das reine Hormon, sondern ein Peptidgemisch, sind jedoch reiner als die durch die geläufige Isolierung gewonnenen Präparate. Die Synthese von Oxytocin und Vasopressin wird noch später bei der Darlegung der Struktur beider Hormone erwähnt werden.

Chemische Eigenschaften des Oxytocins und Vasopressins (5, 6, 10). Nachdem die Reindarstellung beider Hormone gelungen war, wurde ihre Molmasse untersucht, einmal durch Berechnung aus ihren Komponenten nach der chemischen Analyse, zum andern durch osmometrische Bestimmung. Beide Hormone sind *Octapeptide*, wobei *Oxytocin aus Schweine- und Rinderdrüsen dieselbe Zusammensetzung* besitzt, Vasopressin bei beiden Arten sich jedoch durch eine Aminosäure unterscheidet:

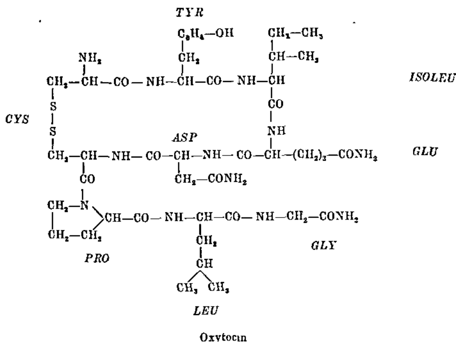
Oxytocin	Molmasse	1007
Vasopressin der Schweinedrüsen .		1056
Vasopressin der Rinderdrüsen .		1084

Bei Oxytocin wurde nach der Beweglichkeit bei der Elektrophorese der isoelektrische Punkt pH 7,7 ermittelt, bei Verwendung einer anderen Pufferlösung wurde der Wert 8,5 gefunden. Für Vasopressin sodann 10,85—10,9 (114). Bei beiden Hormonen stellte man ausgesprochen amphoteren Charakter fest

Nach den Angaben von Du Vigneaud enthalten beide Octapeptide die folgenden Aminosäuren (122):

Aminosäuren	Oxytocin	Vasopressin der Schweinedrüsen	Vasopressin der Rinderdrusen
Leucin	1	—	—
Isoleucin	1	—	—
Tyrosin	1	1	1
Prolin	1	1	1
Glutaminsäure	1	1	1
Asparaginsäure	1	1	1
Cystin	1	1	1
Glycin	1	1	1
Phenylalanin	—	1	1
Arginin	—	—	1
Lysin	—	1	—

Beide Hormone sind also Peptide, bestehend aus acht Aminosaureresten, und unterscheiden sich dadurch, daß Oxytocin *Leucin* und *Isoleucin* enthält, Vasopressin hingegen nicht. Das Vasopressin der Schweinedrüsen enthält an Stelle dieser zwei Aminosäuren *Phenylalanin* und *Lysin* (der sog. *Lysin*-Typ des Vasopressins), Vasopressin der Rinderdrusen dann *Phenylalanin* und *Arginin* (der sog. *Arginin*-Typ des Vasopressins).

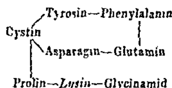


Oxytocin

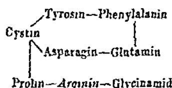
Mit der Strukturaufklarung des *Oxytocins*, insbesondere hinsichtlich der Aminosäurenfolge, beschäftigte sich bei reinen Präparaten namentlich Du Vigneaud mit Mitarbeitern (134), ferner dann Tuppy (119). Im Jahre 1953 wurde von beiden Arbeitsgruppen die vollige Oxytocinstruktur mitgeteilt, s. S. 519.

Es handelt sich also um ein *cyclisches Octapeptid*, wo der vom Cystinrest gebildete Ring insgesamt fünf Aminogruppen enthalt, die Seitenkette dann drei Aminosäurereste. In diesem Molekul wurden drei *Amidogruppen* festgestellt, und zwar an den Carboxylgruppen der Glutaminsaure, Asparaginsäure und des terminalen Glycins.

Gleichzeitig ermittelte man die Struktur reiner Praparate von *Vasopressin*, die aus Schweinedrüsen (125) und aus Rinderdrusen (13, 123) erhalten worden waren. Ungefahr gleichzeitig wie für Oxytocin wurden die Strukturformeln für beide Vasopressin-Typen vorgeschlagen:



„Lysintyp" des Vasopressins aus *Schweine*drüsen



„Arginintyp" des Vasopressins aus *Rinder*drüsen

Beide Typen des Vasopressins besitzen im funfgliedrigen Ring *Phenylalanin* an Stelle des *Isoleucins* im Oxytocinmolekül, und in der Seitenkette besitzt das Hormon *aus* den Schweinedrusen *Lysin*, das aus den Rinderdrüsen *Arginin* an Stelle des Leucins im Oxytocinmolekül. Sonst besitzen die Moleküle der beiden Typen des Vasopressins und das Oxytocinmolekül analoge Struktur.

Durch Oxydation des reinen Hormonpräparates mittels Perameisensäure sowie durch Desulfurierung in Gegenwart von Raney-Nickel wurde eine einzige Substanz gewonnen. Durch oxydative Spaltung der Disulfidbindung des Cystins im Hormonmolekul entstehen zwei Cysteinsaurereste, die in ein einziges Peptidmolekül eingegliedert sind. Bei der Desulfurierung entstehen in ahnlicher Weise zwei Alaninreste. Die Aminosäurenfolge im Peptid wurde durch Analyse der Hydrolysenspaltprodukte untersucht, wobei die N-terminalen Aminosäuren nach der Einwirkung von Dinitrofluorbenzol als DNF-Derivate oder nach der Einwirkung von CS_2 als N-Dithiocarboxyderivate gekennzeichnet wurden; hierbei reagiert auch das phenolische Hydroxyl des Tyrosins Es zeigte sich, daß die eingegliederten Aminosäuren durchweg *L-Konfiguration* haben.

Durch Erhitzen des Oxytocins auf 90—100° C eine Stunde hindurch wird ein Molekül Ammoniak in Freiheit gesetzt. Nach Reaktion des Oxytocins mit Brom wurden zwei Substanzen gewonnen, und zwar: ein aus Cysteinsäure und Dibromtyrosin zu-

sammengesetztes Dipeptid und ferner ein Heptapeptid, das die Aminosäuren Asp, $CySO_3H$, Glu, Gly, Ileu, Leu und Pro enthält. Oxytocin wird weder von Carboxypeptidase, Pepsin noch von Papain in saurem Medium gespalten, Trypsin jedoch inaktiviert das Hormon. Nach FRASER (46) beeinträchtigte Arginase weder die Aktivität von Oxytocin noch von Vasopressin (offenbar nur aus Schweinedrüsen), Tyrosinase bewirkt jedoch Inaktivierung. Oxytocin ist in schwach saurem Medium beständig, im alkalischem Bereich tritt Inaktivierung ein.

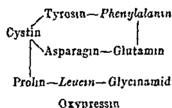
DU VIGNEAUD mit Mitarbeitern (126) führte die *Oxytocinsynthese* durch, und zwar aus p-Toluolsulfonyl-L-isoleucyl-L-glutaminyl-L-asparagin und S-Benzyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamid. Durch Kondensation und Entfernung der Tosylgruppe erhielt er ein Heptapeptid, das sodann mit N-Carbobenzoxy-S-benzylcysteinyl-L-tyrosin kondensiert wurde. Die blockierenden Gruppen an S und N wurden mit Natrium in flüssigem Ammoniak entfernt, und das nonapeptidische Amid wurde hierauf durch Luftoxydation zu dem disulfidischen Octapeptid Oxytocin kondensiert. Das gewonnene Produkt war nach Reinigung durch das Craig-Verfahren sowohl hinsichtlich der chemischen und physikalischen als auch der biologischen Eigenschaften mit dem aus Hypophysen isolierten Oxytocin identisch.

Gewissermaßen eine Modifikation des synthetischen Verfahrens von DU VIGNEAUD ist die neuere Oxytocinsynthese, die RUDINGER (Prag) verwirklichte (95). Die Synthese eines möglichen Zwischenproduktes zur Oxytocindarstellung, des L-Cysteinyl-L-tyrosyl-L-isoleucins beschrieb ROBERTS (94); ein zyklisches Pentapeptid, das Disulfid L-Cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinamid, stellte RESSLER (93) dar und beschrieb seine biologischen Eigenschaften. Das Molekül dieses Peptids unterscheidet sich von Oxytocin durch das Fehlen der Seitenkette: -Prolin-Leucin-Glycinamid. Gleichzeitig wurde ein Analogon dargestellt, das an Stelle des Asparaginylrestes den Rest der α,γ-Diaminobuttersäure enthielt. Da diese Aminosäure nicht zur Synthese benutzt wurde, mußte dieser Rest durch Dehydratation der Amidogruppe des Asparagins zur Nitrilgruppe und durch nachfolgende Reduktion dieser Gruppe entstehen.

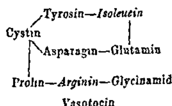
Zwischenprodukte, die an S und N trityliert waren, verwendete VELLUZ (121) bei seiner Oxytocinsynthese; das Verfahren umfaßt insgesamt 33 Reaktionsstufen. Ein anderes Verfahren zur Oxytocinsynthese beschrieb BOISSONNAS (27), der außerdem vier *Analoga des Oxytocins* bereitete, wobei im Molekül der Isoleucinrest durch den Phenylalanin-, Leucin- oder Valinrest ersetzt ist; ferner wurde der Glutaminylrest durch den Asparaginylrest ersetzt. Das synthetisch gewonnene „Valyl"-Analogon des Oxytocins hatte in allen drei biologischen Testen (23) eine dem natürlichen Oxytocin ähnliche Aktivität. Das Leucyl- und Valylanalogon des Oxytocins (Leucyl- oder Valyl-Gruppe an Stelle des Isoleucinrestes) wurde zur selben Zeit unabhängig auch von RUDINGER (96) dargestellt. Nach einem der Oxydation nach DU VIGNEAUD analogen Verfahren wurde die Zyklisierung einer Reihe verschiedener L-Cysteinyl-

-polyglycyl-L-cysteine (54) untersucht. In allerletzter Zeit wurde das *Nonapeptidhomologon* des Oxytocins synthetisch dargestellt, dessen Ring um einen weiteren Tyrosinrest erweitert ist. Dieses synthetische Homologon hemmt die Oxytocinaktivität (BOISSONNAS, R. A. und Mitarbeiter: *Naturwiss.* 44, 632, 1957).

Der Arginintyp des *Vasopressins* wurde in ähnlicher Weise von DU VIGNEAUD (127) synthetisch hergestellt und gleichzeitig auch der Lysintyp, dessen Synthese in letzter Zeit modifiziert wurde (127_{1958}). Zum Vergleich des natürlichen und synthetischen Arginintyps des Vasopressins wurde eine Methode der Verteilungschromatographie ausgearbeitet (31). In allen chemischen und biologischen Eigenschaften konnte Übereinstimmung zwischen den Präparaten des synthetischen und natürlichen Vasopresins ermittelt werden. In allerletzter Zeit wurde ein interessantes Derivat dargestellt; es besitzt ein zyklisches Pentapeptid von der Zusammensetzung wie im Vasopressin und die Tripeptidseitenkette des Oxytocins (72). Das Analogon wurde *Oxypressin* benannt und besitzt schwächere biologische Aktivität als die beiden Hormone. Strukturell stimmt es mit einem der vier von BOISSONNAS dargestellten Oxytocin-Analogen überein, und zwar mit jenem, in welchem die *Isoleucylgruppe* durch Phenylalanin ersetzt ist. Ein zweites derartiges Derivat ist *Vasotocin* vom Arginintyp, dessen Pentapeptid mit der Oxytocinkomponente identisch ist und bei dem die Tripeptidamidkomponente mit der des Vasopressins übereinstimmt. Vasotocin (72_{1958}) besitzt starke Oxytocinwirksamkeit, ist depressorisch wirksam bei Vögeln und besitzt auch Vasopressinwirksamkeit, wie durch biologische Bestimmungen des synthetisch dargestellten Derivates festgestellt wurde. Schließlich ist noch die Synthese des *Isoasparagin*-Analogen des Oxytocins zu erwähnen (LUTZ, W. B. und Mitarbeiter: J. amer. chem. Soc. 81, 167, 1959), das weder Oxytocin- noch pressorische Aktivität besitzt, obwohl seine physikalischen Eigenschaften mit Oxytocin beinahe übereinstimmen.



Oxypressin



Vasotocin

Während das in USP-Einheiten ausgedrückte Verhältnis der pressorischen zur oxytocischen Aktivität je 1 mg bei Oxytocin 7:500 und beim Arginintyp des Vasopressins 600:30 beträgt, wurde bei Oxypressin das Verhältnis 3:20, bei Vasotocin 125:75 gefunden.

Biologische Bewertung der Oxytocin- und Vasopressinpräparate (9). Zur Bewertung von Hypophysenhinterlappenextrakten, die zumeist als Pituitrin be-

zeichnet werden, wendete man einige Methoden zur Erfassung der stimulierenden Wirkung gegenüber der Uterusmuskulatur, ferner der vasopressorischen und antidiuretischen Aktivität an. Daneben wurde bei derartigen Präparaten der Histamingehalt bestimmt. Auch nach Trennung beider Komponenten des Extraktes sind die für die Praxis in Frage kommenden Präparate nicht ganz rein, Oxytocin enthält immer eine bestimmte Menge Vasopressin und umgekehrt. Man stellte allerdings fest, daß auch die einzelnen Hormone qualitativ immer beide Aktivitäten aufweisen, wie bereits erwähnt wurde. Heute sind bereits viele Substanzen bekannt, die die glatte Uterusmuskulatur oder den Blutdruck beeinflussen, daher ist die Spezifität der angewandten Teste für die beiden Hormone sehr wichtig.

Die klassische Methode zur Bestimmung der Oxytocinaktivität ist der *Test am Meerschweinchenuterus* nach DALE und LAIDLAW (38) in vitro. Hierzu dienen Uteruspräparate von 150—250 g schweren Meerschweinchen in der Dioestrusphase, in welcher der Uterus keine spontanen rhythmischen Kontraktionen aufweist. Die Bestimmung der Kontraktionen wird bei dem Präparat in Ringerlösung unter genau angegebenen Bedingungen im Vergleich zu einem Standard vorgenommen. Als eine *Voegtlin-Einheit* wird diejenige Präparatmenge bezeichnet, welche die Aktivität von 0,5 mg des Standardpräparates besitzt, d. h. des sogenannten Voegtlin-Pulvers, das aus Rinderhypophysenhinterlappen dargestellt wird. Im Jahre 1935 wurde von der Kommission für biologische Standardisierung die *internationale Einheit* der oxytocischen Wirksamkeit in analoger Weise bestimmt Das internationale Standardpräparat enthält zwei internationale Aktivitätseinheiten in 1 mg, und zwar sowohl an oxytocischer als auch vasopressorischer Wirksamkeit

Ein anderes Verfahren bestimmt die von einem Oxytocinpräparat ausgelösten Kontraktionen der Uterusmuskulatur in vitro an Präparaten des *Rattenuterus* gemäß HOLTON (65). Ein Uterushorn wird in 10 ml modifizierter Locke-Lösung mit einem verminderten Gehalt an Calciumionen und Glucose bei 32° C suspendiert. Man verwendet das Gewebspräparat von nicht graviden weißen Ratten im Gewicht von ungefähr 120—200 g. Beide Verfahren, sowohl das mit dem Meerschweinchen- als auch das mit dem Rattenuterus, wurden mehrfach modifiziert. Es wurde die Bestimmung der Oxytocinaktivität im Blut geprüft, wobei der Einfluß von Histamin, Adrenalin und Acetylcholin auf das isolierte Uterusgewebe untersucht wurde (70); ferner verfolgte man den Einfluß des verschiedenen Verhältnisses von Oxytocin und Vasopressin in den titrierten Präparaten, weiter den Einfluß verschiedener Mengen an $Mg^{\cdot\cdot}$- und $Ca^{\cdot}$-Ionen, Östrogenen und Progesteron auf die Empfindlichkeit der Bestimmung der Oxytocinwirksamkeit (109). Die Bestimmung von Oxytocin in Gegenwart von Vasopressin wurde an Rattenuteri im Dioestrus vorgenommen (78).

Ein anderes Verfahren der Oxytocintitration verfolgt die *Blutdrucksenkung* bei Hühnern. Diesen Effekt beobachteten PATON und WATSON bereits im Jahre 1912 nach der Injektion von Hypophysenhinterlappenextrakten bei Vögeln; daß es sich

um eine Reaktion auf Oxytocin handelt, erkannte GADDUM im Jahre 1928 (siehe 9). Das Titrationsverfahren arbeitete COON aus (37). Es werden weiße Leghornhähnchen im Gewicht von 1,8—2,2 kg verwendet. Wenn im Präparat eine größere Menge Vasopressin enthalten ist, tritt nach anfänglicher Blutdrucksenkung *sekundärer Anstieg* ein. Auch dieses Verfahren wurde von verschiedenen Autoren mehrmals modifiziert (siehe 9).

Zur Titration der nationalen Standardpräparate wurden alle angeführten Verfahren gleichzeitig nebeneinander untersucht (19, 75, 115); die die Blutdrucksenkung verfolgende Methode ist einfach und am wenigsten kostspielig. Das synthetische Oxytocinpräparat wurde mittels beider Verfahren am überlebenden Uterus sowie auch am Blutdruck bei Hähnchen bewertet (73).

Die Aktivität des *Vasopressins* wird durch Ermittlung der Blutdruckbeeinflussung bei dekapitierten Katzen, anästhesierten Hunden oder Ratten bestimmt (siehe 9) Die *Aktivitätseinheit* wird in analoger Weise wie für Oxytocin definiert, und zwar durch Beziehung auf das internationale Standardpräparat; von diesem enthält 1 mg 2 IE der Vasopressinaktivität.

Die *antidiuretische* Wirksamkeit wird nach BURN (28) an normalen Ratten bestimmt, denen Wasser mittels Magensonde zugeführt wurde. Männliche Ratten im Gewicht von 120—240 g ließ man 12 Stunden hungern; sodann wurden ihnen mittels Magensonde 5 ml warmes Wasser, berechnet auf 100 g Körpergewicht, und ferner das zu überprüfende Hormon in geeigneter Verdünnung subkutan zugeführt. Bei den Versuchstieren wurde die Harnausscheidung eine gewisse Zeit hindurch verfolgt. Der Test wurde nach einigen Tagen mit derselben Tiergruppe gegenüber dem Standardhormonpräparat wiederholt. Die Methode wurde ferner in vielen Modifikationen zur Bestimmung pharmazeutischer Hormonpräparate aus den Hypophysenhinterlappen sowie zur Bestimmung der antidiuretischen Wirksamkeit im biologischen Material verwendet (20, 32, 43, 51, 58, 64, 86, 91, 112, 117). Es wurde auch ein einfaches Verfahren zur Bestimmung an Mäusen beschrieben (59); die Methode wurde ursprünglich von GIBBS ausgearbeitet (50). In letzter Zeit bewährte sich am besten die JEFFERSsche Methode (68) der Bestimmung an Ratten, wobei das Präparat intravenös verabreicht wird. Andere Verfahren verwenden normale hydratierte Hunde oder Tiere mit experimentellem Diabetes insipidus

Biologische Eigenschaften des Oxytocins und Vasopressins (Adiuretin) (1, 3, 4, 7, 8, 8a, 10, 11). Beide Hormone sind, wie bereits erwähnt, im Hypophysenhinterlappen enthalten, es ist jedoch nicht sicher, ob sie auch hier entstehen oder nur gespeichert werden. In letzter Zeit wurden zahlreiche Beweise darüber erbracht, daß auch der *Hypothalamus* beide Hormone enthält (2, 17, 18, 35, 62, 101, 116), und zwar durch Ermittlung der Wirksamkeit der Extrakte bei verschiedenen Tierarten. Nach diesen Anschauungen wäre der Entstehungsort der Hormone der Hypothalamus,

von wo aus die Hormone in die *Pars neurosa* der Hypophyse gelangen Die neurosekretorischen Elemente sind in diesen Geweben verstreut, und diese produzieren beide Hormone, die weitergeleitet und im Hypophysenhinterlappen gespeichert werden. Nach SCHARRER sind nicht die Pituicyten der Pars neurosa die Sekretionselemente, sondern direkt die Nervenzellen.

Beim Vergleich der antidiuretischen Wirksamkeit der aus dem Hinterlappen und dem Hypothalamus isolierten Präparate, insbesondere hinsichtlich der Inaktivierung durch Gewebshomogenate, wurde ihre Verschiedenheit festgestellt (24). Es wurde erwogen, ob Oxytocin und Vasopressin ursprünglich nicht aus einem einzigen Eiweiß- oder Peptidpräcursor entstehen. Mit dieser Untersuchung beschäftigte sich vor allem VAN DYKE; das isolierte Protein sollte dasselbe Verhältnis der oxytocischen, vasopressorischen *und adiuretischen Wirksamkeit aufweisen, wie es ursprünglich in der* Drüse vorliegt. Der Eiweißkörper vom Molekulargewicht ungefähr 30000 wurde der Hydrolyse unterworfen, und neuerdings wurden die darin enthaltenen Aminosäuren mit der Zusammensetzung der beiden Octapeptide Oxytocin und Vasopressin verglichen (120). STEHLE (107) führt die Möglichkeit der gegenseitigen Umwandlung von Oxytocin und Vasopressin an, auch SCHARRER erwägt die Möglichkeit der alleinigen Entstehung von Oxytocin und seine teilweise sekundäre Umwandlung in Vasopressin. Neuerdings wurden diese Fragen erneut durch Anwendung anderer Techniken von ASCHER (14) überprüft und dabei festgestellt, daß Biosynthese und Sekretion von Oxytocin in manchen Fällen bei Ratten unabhängig von der Biosynthese und Sekretion des Vasopressins verlaufen, was für die primäre voneinander unabhängige Existenz beider Hormone spricht.

Die im Hypophysenhinterlappen enthaltene Hormonmenge wurde bei verschiedenen Tierarten und auch beim Menschen untersucht (63) In Schweinehypophysen wurde ungefähr die doppelte Oxytocinmenge wie in Rinderhypophysen aufgefunden (110). Auch *ist das Verhältnis der Hormonaktivitäten* im Hypothalamus und im Hinterlappen bei den verschiedenen Tierarten unterschiedlich, am höchsten beim Hund und am niedrigsten beim Rind

Die Hormonsekretion wird von Nervenreizen gesteuert. Die Hypophysen von im *Dunkeln gehaltenen weiblichen Ratten enthielten mehr Oxytocin, als bei den im* Licht aufgezogenen Tieren ermittelt wurde (102); bei den übrigen Hypophysenhormonen *wurde die umgekehrte Beziehung festgestellt. Einwirkung von Adrenalin* auf die Gehirnrinde im frontoparietalen Teil löste Oxytocinausschüttung bei Kaninchen aus, die an Kontraktionen der Uterusmuskulatur verfolgt wurde (29).

Im menschlichen Blut wurde die Oxytocinaktivität von 1 IE in 100 ml sichergestellt. Es wurden zwei Substanzen aufgefunden, welche die Kontraktionen der Uterusmuskulatur beeinflussen, und zwar auch bei Frauen außerhalb der Schwangerschaft, wo der Gehalt an diesen Substanzen stark schwankt (56). Nur eine von den beiden Substanzen wird von Natriumthioglykolat inaktiviert (s. auch ROBERTSON,

P. A., Hawker, R. W.: *Nature* 180, 343, 1957). Die andere Substanz war auch in Hypothalamusextrakten enthalten. Der Gehalt an beiden Substanzen mit oxytocischer Wirksamkeit im Blut ist während der Schwangerschaft hoch. Im Blut von Schwangeren wurde ein Enzym festgestellt, das die Aktivität des Oxytocins angreift und *Oxytocinase* oder *Pitocinase* genannt wurde. Der Enzymgehalt wurde im Blut zu verschiedenen Zeiten vom Beginn der Schwangerschaft an verfolgt (87); die Plazenta enthält dieses Enzym ebenfalls in hoher Konzentration, hingegen konnte es im Fruchtwasser nicht festgestellt werden. Die Oxytocinase hebt am graviden Uterus den Einfluß des Oxytocins auf; Chinin bewirkt angeblich Hemmung des Enzyms, während man mit Ergometrin oder Prostigmin in dieser Richtung keine eindeutige Ergebnisse erzielte (129). Das Enzym wurde auch aus Schweineovarien isoliert und einige seiner Eigenschaften beschrieben (47), ferner aus Extrakten der Rindermilchdrüse (130). Rattengewebe wurde auf die Fähigkeit der Oxytocininaktivierung systematisch untersucht; während der Gravidität steigt die Aktivität der Oxytocinase vor allem im Uterus bedeutend an (99).

Während der Schwangerschaft wurde bei Frauen nur schwache oxytocische Aktivität im Amnionwasser festgestellt, bei der Geburt steigt jedoch die Aktivität stark an (84). Die Arbeit der Uterusmuskulatur wird von dem antagonistischen System *Oxytocin-Oxytocinase* und dem ihm untergeordneten System *Cholinesterase-Acetylcholin* gesteuert. Oxytocin hemmt offenbar die Cholinesterase und sensibilisiert so den Muskel gegenüber Acetylcholin (129). Der Uterus ist gegenüber Oxytocin am empfindlichsten vor der Geburt und unmittelbar danach.

In letzter Zeit wurde die Beziehung der Hinterlappenhormone zur Sekretion einiger Hormone des Hypophysenvorderlappens, vor allem von ACTH, sehr eingehend untersucht. Bei normalen Tieren beobachtete man nach Verabfolgung beider Hormone des Hinterlappens Anregung der Nebennierenrinde, die jedoch bei hypophysektomierten Tieren nicht eintritt (80). Der für die Anregung der ACTH-Ausschüttung verantwortliche Faktor scheint mit Vasopressin nicht identisch zu sein (92, 100, 111).

Neuerdings wird der Beweis erbracht, daß Oxytocin die Sekretion des luteotropen Hormons (Prolactins) im Hypophysenvorderlappen fördert (22, 39). Der stimulierende Einfluß von Hypophysenhinterlappenextrakten auf die Milchabsonderung bei Tieren während der Lactation ist bereits lange bekannt, erst in letzter Zeit, und zwar nach der Isolierung beider Hinterlappenhormone im Reinzustand, war es jedoch möglich, diese Frage genauer zu entscheiden. Diese Aktivität besitzt vor allem Oxytocin, Vasopressin hingegen nur in geringerem Ausmaß. Zur Hervorrufung derselben Wirkung auf die Milchabsonderung bei Kaninchen sind 17—20 IE Oxytocin erforderlich; dies kommt der Wirkung von 100 IE gleich. Vasopressin besitzt also $1/_5$ der Wirksamkeit des Oxytocins bei der Beeinflussung der Milchabsonderung bei Kaninchen während der Lactation. Die Wirkung des Oxytocins wurde auch bei Ziegen und

Schafen festgestellt (15); bei Kühen untersuchte man die Beziehungen des zugeführten Oxytocins zur Blockierung des endogenen Oxytocins unter Berücksichtigung der Anregung der Milchabsonderung (40).

Das menschliche Blut enthält ungefähr 0,01 IE Vasopressin, berechnet auf 100 ml. Das Hormon wird vor allem in Leber und Niere aus dem Kreislauf ausgeschieden (42, 57, 97, 108); das Inaktivierungsvermögen der Leber wurde herabgesetzt, wenn den Versuchskaninchen vor der Verabfolgung von Vasopressin Wasser in größerer Menge peroral zugeführt wurde (82). Zellfreie Leberextrakte enthalten ein Vasopressin inaktivierendes Enzymsystem; ähnliche Extrakte aus Milz, Nieren und Blut waren weniger wirksam (25). Die Leberextrakte epinephrektomierter Ratten waren in stark vermindertem Maße befähigt, Vasopressin zu inaktivieren.

Die Identität des Hypophysenadiuretins und -Vasopressins steht heute bereits außer Zweifel, ungeklärt bleibt jedoch das Problem bei den antidiuretischen Faktoren des Blutserums und Harns. MIRSKI (81) schließt aus, daß der Blutfaktor in der Hypophyse entsteht, denn nach Hypophysektomie bei den Versuchstieren sank der Gehalt des Faktors im Plasma nicht ab. Die sogenannte „stabile" antidiuretische Substanz, die im Blutserum bei einer Reihe verschiedener Tierarten festgestellt wurde, konnte als *5-Oxytryptamin* identifiziert werden (41). Die Nieren besitzen die Fähigkeit, überschüssige Adiuretinmengen durch den Harn auszuscheiden (33). Zur Isolierung von *Adiuretin aus Harn wurden verschiedene Methoden verwendet, von denen sich einige* zu seiner Isolierung aus dem Hypophysenhinterlappen bewährt hatten (69, 113); zumeist verwendet man das Prinzip der Adsorption. J. HELLER (60) wies nach, daß die Sekretion von Adiuretin des Hypophysenhinterlappens von der Ausscheidung der Substanz im Harn reflexbedingt ist. Nach Inkubation mit Thioglykolat ging diese Wirkung verloren.

Während der Schwangerschaft werden die antidiuretische Aktivität des Blutserums und gleichzeitig die Fähigkeit des Serums, Vasopressin zu inaktivieren, erhöht (34); nach der Geburt stellen sich wiederum die normalen Werte rasch ein. Neben den mineralotropen Corticoiden ist Vasopressin der wichtigste Faktor für den Wasser- und Elektrolyt-Stoffwechsel des Organismus. Vasopressin erhöht normalerweise in physiologischen Dosen die Salzdiurese beim Menschen nicht, ausgeprägt ist jedoch seine Wirkung auf die Wasserretention im Organismus. Für diese Wirkung des Adiuretins wurden drei mögliche Mechanismen in Erwägung gezogen: vor allem unmittelbare Wirkung auf die Nieren durch die Blutbahn, ferner Einwirkung auf die Gewebe und schließlich Beeinflussung des Zentralnervensystems. In letzter Zeit wurde die Möglichkeit aufgegriffen, den gegenseitigen Einfluß von Adiuretin und den Na^{+}-Retention bewirkenden Hormonen auf die Reabsorption in den Nierentubuli zu erforschen. Als Modellversuch diente das permeable System der Froschhaut (30). Insbesondere verfolgte man die Beziehung des Einflusses von Cortison zum Wirkungsmechanismus des Adiuretins auch bei Ratten, die in der ersten Versuchs-

periode eine größere Wassermenge erhielten (74); Cortison erwies sich hier ohne Einfluß.

Die vasopressorische Wirkung des Vasopressins wird durch Verengerung des Lumens in den peripheren Gefäßen bewirkt. FRIEDMANN (48) untersuchte in einer Serie von Arbeiten den Einfluß des Vasopressins auf die durch Cortexon- und Cortisolzufuhr bei Ratten ausgelösten experimentellen Hypertensionen. Nach wiederholter *täglicher Verabreichung von Vasopressin zeigte sich kumulierte depressorische Wirkung*, ähnlich auch bei der durch teilweise Nephrektomie und Abbinden der Niere hervorgerufenen Hypertension. Oxytocin besitzt keine derartige Wirkung. Bei großen Dosen Vasopressin beobachtete der erwähnte Autor *bei hypertensiven sowie bei normalen Ratten eine typisch zweiphasige Wirkung* auf die $Na^{\cdot}$-Toleranz. Bei Versuchen an perfundierten Gefäßen des isolierten Hinterbeins beim Kaninchen wurde festgestellt, daß sich vasokonstriktorische Aktivität des Hormons durch die Konzentration einiger Ionen in der Perfusionsflüssigkeit beeinflussen läßt (118). Niedrigere Konzentrationen von $Ca^{\cdot\cdot}$ und $Mg^{\cdot}$ setzen die Empfindlichkeit herab, ebenso ist das Verhältnis $Na^{\cdot}/K^{\cdot}$ von bedeutendem Einfluß. Ferner untersuchte man die Mitwirkung von Vasopressin an der Entstehung von Ödemen und die biologische Interaktion mit Aldosteron (61).

Der synthetisch dargestellte Arginin- und Lysintyp des Vasopressins wurde in seinen Wirkungen mit dem natürlichen Hormon verglichen, und zwar insbesondere hinsichtlich der Beeinflussung des Gehaltes an Plasmacorticoiden beim Menschen (79). Das Präparat vom Lysintyp ist aktiver als das Präparat vom Arginintyp, an*sonsten wurden in der biologischen Aktivität keine Unterschiede festgestellt.* Bei Oxytocin und Vasopressin wurde hohe Strepogeninwirksamkeit ermittelt (133).

Die Unterschiede in der Stabilität der natürlichen Hypophysenhinterlappenhormone und der synthetischen Peptide untersuchte K. ADAMSON mit Mitarbeitern (Endocrinology 63, 679, 1958); für Oxytocin wurde die maximale Stabilität bei pH 3 festgestellt, und zwar sowohl für das natürliche als auch das synthetische Peptid. Die Stabilität des Vasopressins vom Lysin- und Arginintyp war jedoch verschieden.

Therapeutische Verwendung von Oxytocin und Vasopressin. Beide Hormone *werden bereits seit längerer Zeit in der therapeutischen Praxis verwendet,* anfänglich in Form gereinigter Hypophysenhinterlappenextrakte, die unter der Bezeichnung *Pituitrin* in den Handel kamen Ihr hauptsächliches Anwendungsgebiet war beim *Geburtsakt zur Unterstützung der Uteruskontraktionen,* bei Blutungen post partum; die vasopressorische Komponente kam dann bei Diabetes insipidus zur Anwendung, zur Verhütung des Operationsschocks, bei postoperativen Darmparesen und gemeinsam mit Adrenalin bei Asthma bronchiale Einen großen Fortschritt bedeutete die Abtrennung beider Komponenten, da die pressorische Wirkung bei der Verwen-

dung von Oxytocin unerwünscht ist. Vasopressin kann Ischämie der Uterusmuskulatur, begleitet von Tonussenkung, bewirken (98).

Günstige Ergebnisse erzielte man bei der *intravenösen Applikation* von Oxytocin durch die Tropfeninfusion mit 5% Glucoselosung; in anderen Fällen erzielt man eine ähnliche Wirkung durch wiederholte i.m. oder s.c. Injektionen des Hormons (103). Man versuchte, das Hormon gemeinsam mit 7,5% Alkohol in 5% Glucose durch dieselbe Kanüle, jedoch aus zwei Flaschen i.v. zu verabfolgen (131). Eine gewisse Gefahr der i.v. Hormonzufuhr erblickt man in der Bedrohung des Kindes durch Anoxie oder intrakranielle Blutung. Die optimale Konzentration des Hormons ist hier 1:1000—5000 (77). Intravenöse Applikation von Oxytocin wurde auch bei durch Toxämie komplizierten Geburten untersucht. Das Hormon wurde in der Konzentration 1:2000 in 5% Glucose, und zwar 30—40 Tropfen in der Minute verabreicht (89) PAGE (88) veröffentlichte die Ergebnisse dieser Oxytocinapplikation bei 3500 Geburten, bei denen keine Komplikationen zu beobachten waren. Ungefähr die Hälfte der verabreichten Gabe wird im Serum ungefähr binnen 90 Sekunden inaktiviert (88). Bei Verwendung von synthetischem Oxytocin, das die Schweizer Firma Sandoz unter dem Namen *Syntocinon* einführte, wurden mit dem natürlichen Hormon übereinstimmende Ergebnisse erzielt (16, 26, 44, 73). Auch wurde das natürliche Hormon zur Förderung der Milchausscheidung während der Lactation klinisch geprüft. Mit Verabreichung von 0,5 IE intravenös oder 2 IE i m. erzielte man günstige Ergebnisse (83)

Vasopressin in Form des Tannats zeigt protrahierte Wirkung, besonders wenn es in Erdnuß- oder Rizinusöl appliziert wird (132). Ölige Suspensionen des Tannats kamen in 167 Fällen funktioneller Uterusblutungen durchweg mit Erfolg und ohne Nebenwirkungen zur Anwendung (21). Andere Präparate mit protrahierter Wirkung werden mit Polyvinylpyrrolidon kombiniert; insbesondere konnte so bei der Behandlung von Diabetes insipidus protrahierte antidiuretische Wirkung erzielt werden (53, 76, 105).

Die Verabfolgung des öligen Präparates von Vasopressin-tannat längere Zeit hindurch an Patienten mit Herzkrankheiten rief oft Dekompensation mit Ödembildung hervor, nach dem Aussetzen des Präparates trat jedoch rasch Normalisierung ein (45). Bei sechs von zehn dekompensierten Herzkranken beobachtete man normal erhöhte adiuretische Aktivität (52)

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24. Choriongonadotropine

Außer den bereits erwähnten Hypophysengonadotropinen existieren noch andere Hormone, welche die Gonadenfunktion bei den Säugern anregen, jedoch nicht von der Hypophyse herrühren. Diese Hormone wurden im Harn und Blutserum schwangerer Frauen und trächtiger Tiere gefunden, werden von der äußeren Embryohülle, dem Chorion, sezerniert und daher als *Choriongonadotropine* bezeichnet.

Praktische Bedeutung besitzen vor allem das Serumgonadotropin trächtiger Stuten und das Choriongonadotropin aus dem Harn schwangerer Frauen. Beide Hormone sind ähnlich wie die zwei Hypophysengonadotropine Glykoproteine. Das Choriongonadotropin des Frauenharns wurde bereits in kristallinem Zustand dargestellt. In der Praxis finden sich die erwähnten Hormone öfter als Hypophysengonadotropine, da sie relativ leichter zugänglich sind. In ihren biologischen Wirkungen stimmen sie nicht miteinander überein. Das Serumgonadotropin trächtiger Stuten ähnelt in seiner Wirkung dem Hypophysen-FSH, während das Choriongonadotropin aus Frauenharn wiederum dem Luteinisierungshormon der Hypophyse ähnlich ist.

In der älteren Literatur wurden die biologischen Unterschiede nicht deutlicher hervorgehoben, erst nach der Isolierung der reinen Hypophysengonadotropine wurden auch auf diesem Gebiet weitere Erkenntnisse gesammelt. Dies hing auch in beträchtlichem Maße von der Ausarbeitung biologischer Testmethoden für die einzelnen Gonadotropine zusammen. Die verschiedene biologische Wirkung der einzelnen Gonadotropine wurde bei infantilen und hypophysektomierten Ratten (44) beobachtet, und es erwies sich daher als notwendig, die chemische Zusammensetzung der einzelnen Hormone zu vergleichen und ihre therapeutischen Indikationen zu revidieren.

FSH besitzt einen niedrigeren Gehalt an der Zuckerkomponente und einen höheren Stickstoffgehalt als das Choriongonadotropin des Pferdeserums, es unterscheidet sich ferner durch den Wert des isoelektrischen Punktes und durch andere Konstanten. Das Luteinisierungshormon der Hypophyse besitzt gegenüber dem Choriongonadotropin aus Schwangerenharn einen höheren Stickstoffgehalt und unterscheidet sich durch die Zuckerkomponente, der Gehalt an Hexosamin ist fast derselbe. In der Molmasse stimmt das Choriongonadotropin aus Schwangerenharn mit dem Luteinisierungshormon aus Schweinehypophysen überein. Hierbei ist auf die notwendige Unterscheidung der Serum- und Harnchoriongonadotropine, die in diesem Material *während der Gravidität* aufgefunden werden, von den Gonadotropinen des Serums und Harns *außerhalb der Gravidität* aufmerksam zu machen, da diese von der Hypophyse herrühren.

Gonadotropin aus dem Serum trächtiger Stuten. Mit diesem Hormon beschäftigten sich unabhängig voneinander COLE und HART (16) und ZONDEK (60) erstmals im Jahre 1930. Das Hormon tritt im Stutenblut zwischen dem 37. und 47. Tage der Trächtigkeit auf, und seine Konzentration erreicht zwischen dem 50. und 80. Tag das Maximum. CATCHPOLE und LYONS (11) stellten fest, daß dieses Gonadotropin vom Chorionepithel sezerniert wird und mit der Sekretion des Hypophysenvorderlappens nicht zusammenhangt. In der angelsächsischen Literatur wird es mit der Abkürzung **PMSG** bezeichnet (Pregnant Mare Serum Gonadotropin).

Die ersten Versuche zur Reinigung dieses Hormons beschrieben Goss und COLE (25) im Jahre 1931, gemeinsam mit einigen Angaben uber seine physikalisch-chemischen fur die Hormondarstellung wichtigen Eigenschaften. Es wurde der Versuch angestellt, das Hormon durch Fällung mit Aceton und Alkohol bei verschiedenem pH zu gewinnen, und man erzielt auf diese Weise eine bis 1800fache Hormonkonzentration gegenuber der Konzentration im Serum (10). Durch Modifizierung dieses Verfahrens gelangte man später zu einem Praparat, das 700 IE in 1 mg enthielt (26).

RIMINGTON und ROWLANDS (51) erzielten ein Präparat mit 12500 IE/mg. Hierbei wurde das Serum trächtiger Stuten mit demselben Volumen Wasser verdünnt und der pH-Wert mit Phosphorsäure auf 3,6 eingestellt. Der ausgefallene Niederschlag wurde zentrifugiert und gewaschen, die vereinigten Filtrate dann neutralisiert und Adsorption an in der Lösung gefallter Benzoesaure vorgenommen. Das Adsorbat wurde in Aceton und der Ruckstand sodann in Wasser bei pH 7,0 gelost. Aus dieser waßrigen Lösung erhielt man dann das Hormonpräparat durch Fraktionierung mit Alkohol. Aus 10 l Serum wurden ungefähr 36 mg Hormon dargestellt. Aus der Bestimmung der Beweglichkeit bei der Elektrophorese wurde der isoelektrische Punkt des Hormons bei pH 2,6—2,65 ermittelt. Molekulargewicht beträgt cca 30000.

Durch spatere Versuche der Fraktionierung mittels Papierelektrophorese (53) wurden drei Komponenten festgestellt, die sich bei den biologischen Testen qualitativ abweichend verhielten; außer der follikelstimulierenden Komponente wirkten zwei weitere Komponenten auch auf das interstitielle Gewebe. Bei Zonenelektrophorese an Stärke (6) wurde der Eiweißkomplex in mehrere Komponenten zerlegt, von denen eine voll wirksame den isoelektrischen Punkt pH 1,8 besaß, also abweichend von dem ursprunglichen Wert des Präparates von 2,65.

Fur den ursprunglichen aus dem Serum isolierten Eiweißkomplex wurden einige physikalische und chemische Eigenschaften ermittelt (38, 24) Bei der Analyse der Präparate stellte man die folgenden Werte fest:

N	10,6 %	*Tyrosin*	3,54 %
S	0,85 %	*Lysin*	8,8 %
Amino-N	0,46 %	*Histidin*	3,25 %
Galactose	14,1—17,6 %	*Tryptophan*	1,37 %
Hexosamin	8,4 %	*Arginin*	2,1 %

Es zeigte sich, daß der Gehalt an der Zuckerkomponente in den verschiedenen Präparaten kein Maß für die biologische Wirksamkeit des Hormons darstellt. Die einzelnen Autoren benützten verschiedene Präparate, so daß in den Analysenergebnissen keine völlige Übereinstimmung besteht.

Eine neue Methode der Hormondarstellung beschrieb J. LEGAULT-DÉMARE mit Mitarbeitern (Biochim. biophys. Acta 30, 169, 1958). Durch Fraktionierung mit Äthanol gewinnt man ein Präparat mit der Aktivität von 250—300 IE/mg, durch Chromatographie an Permutit und weitere Reinigung wurde eine Fraktion mit der Wirksamkeit 1000—2000 IE/mg isoliert; durch wiederholte Adsorption der das Hormon begleitenden Eiweißballaststoffe an Bariumcarbonat erhielt man schließlich ein Präparat mit der Aktivität 10000 bis 13000 IE/mg. Die Gesamtausbeute betrug 60—70% der ursprünglichen Plasmaaktivität.

In sauren Lösungen sind die Hormonpräparate ziemlich unbeständig, in alkalischem Medium dann bei erhöhter Temperatur. Die Inaktivierung ist nicht mit Oxydation verbunden, wie sich bei Messungen in Gegenwart von Sauerstoff und Stickstoff bei 60° C und pH 5,9 ergab. Auch in trockenem Zustand wurde eine gewisse Unbeständigkeit der Präparate beobachtet. LI mit Mitarbeitern (38) ermittelte bei einem 1000 IE/mg enthaltenden Präparat nach zwei Wochen Lagerzeit ungefähr die Hälfte der Wirksamkeit, sodann war jedoch das Präparat bereits beständig. Einen ähnlichen Aktivitätsverlust beobachteten auch RIMINGTON und ROWLANDS (51) bei der Aufbewahrung trockener Präparate. Die Ursachen dieser Inaktivierung sind bisher unbekannt.

Wesentliche Aktivitätsverluste wurden auch nach dem Filtrieren von Lösungen dieses Hormons und nach Zusatz verschiedener Konservierungsmittel festgestellt (17). Man untersuchte auch den Einfluß der Zugabe verschiedener Kolloide auf die Aktivität des Serum-Choriongonadotropins (50), wobei man jedoch zu keinen positiven Ergebnissen gelangte; bei ähnlichen Versuchen mit dem Choriongonadotropin aus Frauenharn ergab sich jedoch eine überraschende Steigerung der Wirksamkeit

Sowohl durch Keteneinwirkung als auch durch Formaldehyd oder salpetrige Säure wird die Hormonwirksamkeit rasch zerstört; die freien Aminogruppen sind also für die Hormonaktivität wahrscheinlich unerläßlich. Völlige Inaktivierung des Hormons tritt auch nach längerer Einwirkung von Reduktionsmitteln, wie z. B. von Cystein, ein. Es handelt sich wahrscheinlich um Zerstörung der Disulfidbindungen im Hormon. Auch verschiedene Enzyme vernichten rasch die Wirkung des Hormons; es wurden Pepsin, Trypsin, Papain, Chymotrypsin und Carboxypeptidase untersucht. Abspaltung der Zuckerkomponente aus dem Hormonmolekül führt zur raschen Inaktivierung. Auch Einwirkung von Harnstoff in höherer Konzentration löst Inaktivierung des Hormons aus, die durch Wärme beschleunigt wird. Durch thermodynamische Erwägungen über die Hormoninaktivierung wurde auf diese Weise festgestellt, daß der Verlauf der Inaktivierungsreaktion mit der Charakteristik der klassischen Eiweißdenaturierung übereinstimmt (5)

Die biologischen Eigenschaften des Gonadotropins aus dem Serum trächtiger Stuten werden bei der Besprechung des Choriongonadotropins aus Schwangerenharn kurz behandelt werden.

Gonadotropin aus Schwangerenharn. Das Vorkommen von Gonadotropin im Harn schwangerer Frauen beobachteten Aschheim und Zondek bereits im Jahre 1927 (3). Später ergab sich, daß das Hormon nicht von der Hypophyse, sondern dem Chorion herrührt und daß die maxmiale Abscheidung des Hormons zwischen dem 60. und 80. Tage der Schwangerschaft erfolgt. Die Ermittlung der Sekretion dieses Hormons bzw. seiner Konzentration im Harn dient als relativ einfacher Schwangerschaftsnachweis. In der angelsächsischen Literatur wird das Hormon als HCG (Human Chorione Gonadotropin) bezeichnet.

Es wurden viele Versuche zur Anreicherung dieses Hormons unternommen, insbesondere durch fraktionierte Fallung mit Alkohol und mit Hilfe von Adsorptionsverfahren, vor allem unter Verwendung von Benzoesäure als Adsorbens. Gurin (27) beschrieb im Jahre 1939 die Darstellung eines sehr reinen Präparates, das sich bei der Elektrophorese, in der Ultrazentrifuge und bei der Diffusionsmessung in Lösungen einheitlich verhielt. Der gesammelte Harn wurde mittels Benzoesaure adsorbiert und das Adsorbat dann mit Aceton extrahiert. Der unlosliche Trockenrückstand (500 mg aus je 1 l Harn) wurde hierauf mit 50 %igem Alkohol bei pH 6,0 extrahiert und mit absolutem Alkohol in der Kälte gefällt. Auf je 1 l Harn wurden nach dem Trocknen 8 mg Niederschlag erhalten. Nach Auflösen in einer kleinen Menge kaltem Wasser wurde die Lösung auf pH 5,0 gebracht und mit Chloroform ausgeschüttelt. Die Emulsion wurde sodann mit Wasser gewaschen und die klare Losung nach der Dialyse verdampft. Man erhielt ein Präparat mit 6000 IE in 1 mg, wobei die Ausbeute ungefähr 6 mg auf 1 l Harn betrug.

Katzman und Mitarbeiter (30) gelangten zu einem ähnlich wirksamen Präparat nach einem Verfahren, bei welchem der Schwangerenharn mit Essigsäure auf pH 3,5 angesäuert und nach dem Filtrieren das klare Filtrat an einer Permutitsaule adsorbiert wurde. Die Saule wurde mit kaltem Wasser gewaschen und das Hormon sodann mittels 38 % Alkohol mit einem Zusatz von 10 % Ammoniumacetat eluiert. Weiter wurde Alkoholfallung vorgenommen und die Fraktion zwischen 70—75 % Alkoholkonzentration gesammelt. Der Niederschlag besaß nach dem Trocknen eine Wirksamkeit von 8500 IE/mg.

Ein Verfahren zur Gewinnung eines *kristallisierten* Hormonpraparates wurde im Jahre 1948 von Cleasson und Mitarbeitern (14) beschrieben; hierbei arbeitete man durchweg bei +4° C. Das Hormon wurde aus Harn an Benzoesäure adsorbiert, das Adsorbat nach dem Trocknen mit Aceton zur Entfernung der Benzoesaure extrahiert und der Rückstand dann mit M/15 Acetatpuffer (pH 4,8) eluiert und sodann Alkohol bis zur Konzentration 85 % hinzugefugt. Der derart gewonnene Niederschlag wurde

wiederum mit Acetatpuffer extrahiert und die Fällung mit Alkohol wiederholt. Aus 1 l Harn erhielt man 25—30 mg Hormon mit der Aktivität 4000—6000 IE/mg. Die Lösung dieses Präparates wurde hierauf durch Zutropfenlassen einer Protaminlösung von Ballaststoffen gereinigt und das Filtrat dann mit Alkohol stufenweise bis zu 50 und 60% Konzentration gefällt. Der langsam ausfallende Niederschlag kristallisiert innerhalb 24 Stunden in dünnen nadelförmigen Drusen. Bei der Bestimmung der Homogenität ergab sich, daß das Präparat, auch wenn es kristallin vorliegt, nicht einheitlich ist.

Ein verhältnismäßig einfaches Verfahren zur Darstellung von Choriongonadotropin *aus Schwangerenharn beschrieb in letzter Zeit* LANDGREBE *mit Mitarbeiter* (34); hierbei wurde wiederholt an Benzoesäure adsorbiert; nach deren Beseitigung aus dem Adsorbat mit Aceton erhielt man nach dem Trocknen ungefähr 200 mg Rückstand auf je 1 l Harn. Sodann wurde mit Acetatpufferlösung vom pH 4,8 extrahiert, mit Aceton fraktioniert und die Fraktion zwischen 45—70% Acetonkonzentration in der Lösung genommen. Der Niederschlag enthält ungefähr 70% der ursprünglichen Gesamtaktivität. Nach dem Auflösen wurde die Acetonfraktionierung wiederholt und die Fraktion zwischen 45—60% Acetonkonzentration genommen. Im Niederschlag wurden ungefähr 45% der ursprünglichen Wirksamkeit gewonnen. Das Präparat besaß 4000 IE/mg.

Ein chromatographisches Verfahren zur Isolierung des Hormons unter Verwendung des Ionenaustauschers Decalso F und einer Säule mit Tricalciumphosphat beschrieb neuerdings BUTT mit Mitarbeitern (9). Die durch Elution gewonnenen Fraktionen wurden biologisch und analytisch ausgewertet und einige Aminosäurenkomponenten und die Menge der Zuckerkomponente des Hormons angeführt. Bei Papierelektrophorese eines Handelspräparates des Hormons wurden fünf Komponenten festgestellt; eine hiervon enthielt eine Steroidverbindung, wie SCHNEIDER und FRAHM (52) auf Grund verschiedener mit dieser Fraktion durchgeführter Nachweisreaktionen annehmen.

Für die reinsten aus Schwangerenharn gewonnenen Präparate von Choriongonadotropin wurde der *isoelektrische* Punkt bei pH 3,2—3,3 und das Molekulargewicht 100000 ermittelt. Das Hormon enthält 12% Stickstoff und 1,96% Schwefel. Von den Aminosäuren wurden nach der Hydrolyse Tyrosin, Histidin, Arginin und Tryptophan nachgewiesen. Zur Untersuchung der Aminosäuren in den Hydrolysaten von *Choriongonadotropin wurde die Papierchromatographie herangezogen* (59). *Die Angaben der verschiedenen Autoren über den Gehalt der Zuckerkomponente im* Hormonmolekül weichen ziemlich stark voneinander ab. GURIN stellte 10,7% Galactose fest, MICHL (42) gibt bis zu 40% der Zuckerkomponente und 5,2% Hexosamin an, BUTT (9) führt nur 2% Hexose an. Im Hormonmolekül wurden weder Cystin noch Cystein nachgewiesen.

Das Hormon löst sich leicht in Wasser, in organischen Lösungsmitteln ist es jedoch unlöslich. Die Choriongonadotropinlösungen verlieren auch bei niedriger Temperatur

ihre Aktivität. KATZMAN (30) und PEDERSEN (60) stellten fest, daß einige Schutzkolloide die Stabilität des Hormons in Lösungen erhöhen. Durch Erhitzen oder Einwirkung von Säuren oder Laugen nimmt der Aktivitätsverlust des Hormons rasch zu. Getrocknet ist das Hormon zum Unterschied von Serumchoriongonadotropin sehr beständig. BISCHOFF (5) untersuchte die Inaktivierung des Hormons in Lösungen bei verschiedener Temperatur. Eine Lösung von 100 IE in 1 ml 1%igem NaCl verlor bei pH 6,0 nach 5 Minuten. Erhitzen auf 65° C 50% und innerhalb 15 Minuten 76% der Aktivität.

Trypsin, Papain, Chymotrypsin und Ptyalin zerstören die biologische Aktivität des Hormons (1). Pepsin soll angeblich die Aktivität des Choriongonadotropins nicht beeinträchtigen. Man untersuchte die Wirksamkeiten in Ultrafiltraten nach der enzymatischen Spaltung des Hormons durch verschieden lange Einwirkung von Pepsin und Trypsin (23). Die Spaltprodukte zeigten biologische Wirksamkeit. Das Hormon ist bei niedriger Temperatur gegenüber Reduktionsmitteln und gegenüber Inaktivierung durch Keten und salpetrige Säure widerstandsfähiger. Harnstoffzusatz zu den Hormonlösungen bewirkt Aktivitätsverlust infolge Proteindenaturierung (5).

Neuerdings wurden die chemischen und auch die biologischen Eigenschaften der Mucoproteine des normalen Männerharns mit den Mucoproteinen des Schwangerenharns verglichen (31). In beiden Fällen war ungefähr dieselbe Menge von Mucoproteinen vorhanden, der Komplex besaß hinsichtlich des Gehaltes an der Zuckerkomponente ähnliche Zusammensetzung, jedoch nur die aus dem Schwangerenharn isolierte Substanz besaß hohe biologische Wirksamkeit. Beim Vergleich der Hydrolysate von Choriongonadotropin mit den Hydrolysaten des Hypophysengonadotropins aus dem Harn von Frauen nach der Menopause wurde mittels Papierchromatographie eine ähnliche Zusammensetzung festgestellt (21)

Biologische Bewertung der Choriongonadotropine. *Zur biologischen* Titration dieser Präparate können die für Hypophysengonadotropine benutzten Verfahren angewandt werden. Wie bereits erwähnt, ähnelt das Serumgonadotropin trächtiger Stuten in seiner biologischen Wirksamkeit sehr dem Hypophysen-FSH und wird also nach analogen Verfahren titriert (s. S. 441). Neuerdings wurden die einzelnen Faktoren untersucht, welche die Empfindlichkeit der Reaktion beim Test an Ratten beeinflussen (12). Als *internationale Einheit* (IE) wird die Aktivität von 0,25 mg des Standardpräparates von Gonadotropin aus dem Serum trächtiger Stuten bezeichnet. Man untersuchte die Fehlergrenze, die bei der Bestimmung dieses Hormons nach der Methode der Wägung der Ovarien (29) zu berücksichtigen ist, und erhielt einen Wert von 15%.

Die *internationale Einheit* der Wirksamkeit von Choriongonadotropin aus Schwangerenharn ist die Wirksamkeit von 0,1 mg des Standardpräparates, wie auf der 3. Internationalen Konferenz für Hormonstandardisierung in Genf im Jahre 1938 festgelegt wurde. Die verwendeten Titrationsmethoden stimmen mit den Verfahren

fur das Luteinisierungshormon der Hypophyse uberein (s S. 446). Für die klinische Bestimmung des Choriongonadotropins wurde der sogenannte *hyperämische Test* vorgeschlagen, der an 21—26 Tage alten Ratten im Gewicht von 35—50 g durchgeführt wurde. Ein Vorteil dieses Verfahrens ist seine Anspruchslosigkeit und rasche Durchfuhrung (2). Den *Schwangerschaftstest* nach dem Gehalt an Choriongonadotropin im Harn arbeiteten ursprunglich ASCHHEIM und ZONDEK (3) aus. Hierbei wurde bei den Versuchstieren nach Einspritzen des zu untersuchenden Harns die hyperämische Reaktion in den Ovarien verfolgt. Anfanglich wurden Mause verwendet, und die Autopsie erfolgte 36 Stunden nach der Injektion. Der Test wurde später von zahlreichen Autoren modifiziert und zur quantitativen Anwendung vervollkommnet.

Eine andere Testmethode ist ein Verfahren an Fröschen. Es werden Männchen von verschiedenen Arten verwendet, und man verfolgt die Ejakulation nach der Hormonzufuhr. Der Test an Mannchen von *Rana esculenta* ist eine sehr rasche, jedoch nicht allzu empfindliche Methode und daher eher zur qualitativen Bewertung geeignet (33). Die Empfindlichkeit der Reaktion wurde bei Mannchen von *Bufo viridis* (58) untersucht; hierbei stellte man eine jahreszeitbedingte Variabilitat und die hochste Empfindlichkeit im April fest. Das Verfahren mit Männchen der Art *Bufo melanostictus* ist angeblich sehr spezifisch und bei der Darstellung der statistischen Durchschnittswerte auch hinreichend genau (43). Die Empfindlichkeit des Testes hängt auch stark von den Bedingungen ab, unter denen die Tiere gehalten werden; bei *Rana pipiens* bewahrte sich am besten die Temperatur von 6° C, Halten der Tiere bei konstantem Licht und einer bestimmten Diat. Nach Einhalten dieser Bedingungen 14 Tage hindurch sollen die Tiere auf die Reaktivitat untersucht und resistente Tiere ausgeschaltet werden 50 % der Tiere reagieren dann auf die Gabe von 20 E und 100 % auf 30—40 E (32).

Die quantitative Bewertung der Aktivität der Praparate kann ferner mit Hilfe der Wachstumsbestimmung des ventralen Prostatalappens bei infantilen hypophysektomierten Ratten erfolgen. Der auf der Bestimmung des Wachstums der Samenblasen begrundete Test ist fur diesen Test nicht verwendbar, und zwar infolge der allzu großen Standardabweichung, die bei der Verwendung dieses Verfahrens beobachtet wurde (18)

NEUKOMM (46) beschrieb die *polarographische* Bestimmung des Choriongonadotropins und fuhrt hohe Empfindlichkeit der Methode an, so daß noch 0,2 IE der Aktivität bestimmt werden konnen. Das Verfahren beruht auf der Bildung einer Pranatriumwelle am Polarogramm, welche die Eiweißkörper infolge Katalyse der Wasserstoffabscheidung geben Daraus ergibt sich eine gewisse Unspezifitat der Methode, die dennoch zur einfachen informativen Bewertung der Praparate und des Hormongehaltes im Harn nach Fraktionierung mit Alkohol vorteilhaft verwendet werden kann.

CHEYMOL und Mitarbeiter (13) verglichen die einzelnen Verfahren zur Bewertung der Choriongonadotropine namentlich unter Berucksichtigung der Umrechnung der einzelnen Ergebnisse auf internationale Aktivitätseinheiten.

Biologische Eigenschaften der Choriongonadotropine. Diese Hormone werden vom Choriongewebe des graviden Organismus sezerniert (15) und kommen in Blut und Harn vor. Das Choriongonadotropin trächtiger Stuten findet sich in der Konzentration von 43—350 IE/ml Blutplasma, das Choriongonadotropin schwangerer Frauen wird in Mengen bis zu 1000000 Ratteneinheiten täglich ausgeschieden. Nach i.m. oder i.v. Verabreichung konnte das *Hormon* noch nach 7 *Tagen* im *Blut* nachgewiesen werden (36). Nach längerer Verabfolgung großer Dosen werden im Organismus Antikörper gegenüber dem zugefuhrten Choriongonadotropin *beiderlei* Ursprungs gebildet (8, 37, 41, 48, 54). Nach Verabfolgung von Serumchoriongonadotropin wurden *Antikörper im Blut gebildet, die erst nach 15 Monaten verschwanden* (48).

Die Bestimmung von Choriongonadotropin neben Östrogenen in der menschlichen Plazenta einschließlich der Methoden der *biologischen* Auswertung von Extrakten und der statistischen Bearbeitung wurde ausfuhrlich von DICZFALUSY (19) beschrieben. Nach Fraktionierung der Homogenate von Plazentagewebe durch Zentrifugieren ermittelte man $^{1}/_{4}$ der Gesamtaktivität des *Choriongonadotropins in* der Fraktion der Zellkerne, etwas weniger *als* $^{1}/_{4}$ in der Mitochondrienfraktion und mehr als die Hälfte in der Mikrosomenfraktion und der loslichen Zytoplasmafraktion (55). Quantitativ wurde das ausgeschiedene Hormon im Harn nach dem Verfahren der biologischen Titration am Rattenuterus und an den Ovarien *hypophysektomierter* Ratten (40) während des raschen Ansteigens des Hormongehaltes in der Schwangerschaft bestimmt. Es wurden auch qualitative Unterschiede in der Wirkung der Extrakte zu verschiedenen *Zeiten* vom *Beginn* der Schwangerschaft *an festgestellt.*

Mit der histochemischen Untersuchung des Bildungsortes von Choriongonadotropin beschäftigten *sich* in *allerletzter* Zeit K. THOMSEN und R. WILLEMSEN (Acta endocrinol. 30, 161, 1959). Es wurde festgestellt, daß Choriongonadotropin von den *Trophoblastzellen der Basalplatte und der Placentarsepten* ausgeschuttet wird. Im dritten Schwangerschaftsmonat tritt plotzlicher Anstieg der Hormonsekretion und der Aktivitat der Trophoblasten, weniger der Langhanszellen ein.

Die gonadotrope Aktivitat des Hormons aus dem Schwangerenharn *ähnelt* sehr der Wirkung des Luteinisierungshormons der Hypophyse. Es wird dadurch vor allem das interstitielle Gewebe der Ovarien angeregt (28), bei hypophysektomierten Ratten wurde auch Stimulierung der Follikel beobachtet (40, 57). Bei normalen Ratten bewirkt Darreichung von Choriongonadotropin keine Veranderungen in den Nebennieren, bei kastrierten Tieren tritt jedoch ausgepragte Hyperplasie der reticulären Zone auf (39). *Gleichzeitig* verabfolgtes Hypophysen-FSH *erhöht* die Wirksamkeit

des Choriongonadotropins aus Schwangerenharn stark. Bei Verabfolgung eines Gemisches beider Choriongonadotropine an einseitig nephrektomierte Ratten trat Blutdrucksteigerung jedoch nur bei Männchen auf (7). Ähnlich wie die Extrakte der Pflanze *Lithospermum ruderale* bewirken auch einige Chinone und Naphthochinone Hemmung der Gonadotropine, wie sich in vivo bei Ratten ergab. Hydrochinondiacetat setzte die Wirksamkeit des injizierten Serumgonadotropins um 50 % herab (47).

Choriongonadotropine in der klinischen Praxis. Beide Hormone kommen für die praktische Verwendung in Ampullen als Trockensubstanz, das Choriongonadotropin des Harns außerdem auch in Form von Implantationstabletten in den Handel. Beim Vergleich der Anwendung von wäßrigen Lösungen und öligen Suspensionen oder mit Wachszusatz bewährten sich die wäßrigen Lösungen besser (4). Die Implantationstabletten enthalten lyophilisiertes Choriongonadotropin mit Magnesiumstearat. Bei der experimentellen Anwendung von Implantationstabletten konnte protrahierte Hormonwirkung auf die Dauer von 20 Tagen erzielt werden (20). Die normalen Präparate werden nach dem Auflösen subkutan oder intramuskulär verabreicht.

Die Bestimmung von Choriongonadotropin im Harn dient als diagnostischer Test auf die Schwangerschaft, ferner auch bei Chorionepitheliom, da die ausgeschiedenen Hormonmengen noch höher als während der Schwangerschaft sind. Der kombinierte Test mit Verabfolgung von Serumgonadotropin und Choriongonadotropin aus dem Harn wurde diagnostisch zur Unterscheidung von hypergonadotroper und hypogonadotroper Amenorrhöe verwendet (49).

Sonst ist die praktische Verwendung der Choriongonadotropine in der Therapie ähnlich wie bei den Hypophysengonadotropinen FSH und dem Luteinisierungshormon, das Choriongonadotropin aus dem Harn wird jedoch öfter benutzt als das weniger zugängliche Luteinisierungshormon der Hypophyse. Man verwendet es bei Störungen der Hormonregulierung dort, wo die Gelbkörperbildung bei funktionsfähigen Ovarien angeregt werden soll, falls nicht ein hoher Gehalt von Gonadotropinen im Harn festgestellt wird. Die Darreichung von großen Hormongaben längere Zeit hindurch ist zu vermeiden, da auch Ruptur des stark hypertrophierten Ovariums (45) oder Bildung einer Lutealzyste zu verzeichnen waren. Bei Männern verabfolgt man das Hormon bei Eunuchoidismus bei funktionell reaktionsfähigen Testes, wenn im Harn kein hoher Gehalt an Gonadotropinen festgestellt wurde, ferner bei Kryptorchismus, falls dieser nicht auf ein anatomisches Hindernis zurückgeht. Choriongonadotropin wird ferner bei Oligospermie und Azoospermie bei ungenügender Entwicklung des interstitiellen Gewebes der Testes verabreicht. Bei Frauen kommen niedrigere Gaben als bei Männern zur Anwendung.

Choriongonadotropin aus Schwangerenharn wurde mit Erfolg bei verschiedenen Störungen der geschlechtlichen Entwicklung angewandt (22). Über die Bedeutung

der nervösen Steuerung der Ovarialfunktion bei Choriongonadotropinzufuhr sowie über die Aussichten des Verfahrens in der klinischen Praxis berichtete in allerletzter Zeit ŠTĚRBA (56). LAX (35) untersuchte den Einfluß von Choriongonadotropin auf das Bluteiweiß.

Serumgonadotropin wird bei genitalen Hypoplasien angewandt, bei Dystrophia adiposogenitalis, bei Störungen der Spermiogenese und verschiedenen Störungen der Funktion der Ovarien. Ähnlich wie FSH wirkt dieses Hormon nicht auf die Sekretion der Androgene des interstitiellen Gewebes der Hoden bei Mannern und auf die Sekretion der Östrogene bei Frauen.

Einige Handelspräparate von Choriongonadotropinen

Gonadotropin aus dem Serum trächtiger Stuten

ANTERON	Schering BR	
ANTEX	Leo Dänemark	
APOIDINE	Parke Davis USA	
EQUOMAN	H. Mack, BR	
GESTYL	Organon Holland	
GONADOGEN	Upjohn USA	
PREDALON-S	Orgapharm BR	
SEROGAN	BDH England	
SEROGONADIN	Prolek, Beograd, Jugoslawia	

Choriongonadotropin aus Schwangerenharn.

ANTELOBINE	Byla Frankreich	
ANTREGONE	Abbott USA	
ANTUITRIN S	Parke Davis USA	
A.P.L	Ayerst Lab. USA	
CHORIOMAN	Mack BR	
CHORIONIC GONADOTROPHIN	Armour USA	
FOLLUTEIN	Squibb USA	
GLANDUANTIN	G. Richter Ungarn	
GONABION	AWD BR	
NEO APOIDINE	Parke Davis USA	
PHYSEX	Leo Dänemark	
PRAEDYN	Spofa ČSR	
PRANTURON	Schering USA	
PREDALON	Orgapharm BR	
PREGNYL	Organon Holland	
PRIMOGENYL	Schering BR	
PROLAN	Bayer BR	
SYNAPOIDIN	Parke Davis USA	(+ FSH)

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s. S. 457.

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25. Insulin und Glucagon

Obwohl Versuche zur Pankreasentfernung bei Tieren bereits viel früher durchgeführt wurden, verglichen erst KLEBS und MUNK im Jahre 1869 die Erscheinungen nach Pankreasexstirpation mit der bereits damals gut bekannten Zuckerkrankheit (Diabetes mellitus). Infolge unvollkommener Entfernung der Drüse ist es jedoch nicht gelungen, den unmittelbaren Zusammenhang zu beweisen. MEHRING und MINKOWSKI führten dies im Jahre 1890 an Hunden mit Erfolg durch und erbrachten so den ersten Beweis über die Beziehung der Pankreasfunktion zu dieser Krankheit. Durch Transplantationsversuche bei pankreasektomierten Hunden wurden verschiedene Symptome beseitigt, die mit dem Verlust der Pankreasfunktion zusammenhängen; auf diese Weise konnte der Zusammenhang dieser Drüse mit der Zuckerverwertung im Organismus bestätigt werden. Zu Beginn dieses Jahrhunderts erwies GLEY, daß durch Unterbrechung des Blutkreislaufs beim Versuchstier Symptome von Diabetes auftreten. Zu dieser Zeit war bereits die Bildung von Enzymen im Pankreas bekannt, die für die Verdauungsprozesse im Verdauungstrakt erforderlich sind, und es wurde der Fehlschluß gezogen, daß der Zuckerstoffwechsel von demselben Gewebe gesteuert wird, das diese Enzyme abscheidet.

LANGERHANS beschrieb zwar bereits im Jahre 1869 das im Pankreas verbreitete Epithelgewebe, das sich von dem Gewebe unterscheidet, von welchem der Verdauungssaft in den Verdauungstrakt abgeschieden wird; seine eigentliche Funktion wurde jedoch noch nicht erkannt. Diese Inseln des Epithelgewebes wurden nach jenem Forscher benannt; ihre Bedeutung für den Kohlenhydratstoffwechsel wurde gegen Ende des vergangenen Jahrhunderts erkannt. Nur diese Inseln sind Bestandteile des endokrinen Systems, während das übrige Pankreasgewebe nicht endokrin wirksam ist.

Die kanadischen Forscher BANTING und BEST (38) bereiteten den ersten hormonal wirksamen Extrakt aus Pankreas; die Drüse wurde zunächst an ihren Ausführungsgängen unterbunden, bis das fermentbildende Gewebe atrophierte, während die LANGERHANSschen Inseln unversehrt blieben. Auf diese Weise wurde der störende Einfluß der Enzyme beseitigt und die Extrakte setzten erwartungsgemäß den Blutzuckerspiegel nach Verabfolgung an Diabetiker herab. Das in den Pankreasinseln vorausgesetzte Hormon wurde noch vor seiner Isolierung *Insulin* genannt. Das kristallisierte Hormon wurde erstmals von ABEL im Jahre 1927 gewonnen; es wurde festgestellt, daß es Eiweißcharakter besitzt. Seine Struktur und vollständige Aminosäurenfolge in den Peptidketten klärte SANGER mit Mitarbeiter im Jahre 1952 auf.

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gelost und das Hormon nach Einstellung des pH im isoelektrischen Punkt gefallt. Man erhält so ein amorphes Insulinpräparat.

Ein kristallisiertes Hormonpraparat wurde erstmals von ABEL (33) dargestellt, und zwar durch vorsichtige Fällung des Hormons aus Acetatpuffer durch allmähliches Hinzufugen schwacher Basen bis zum pH 5,6 der Losung. ABEL ging von einem amorphen Insulinpräparat mit ca. 13 IE/mg aus und erhielt aus 1 g dieses Praparates insgesamt 260 mg kristallisiertes Präparat mit der Aktivitat 22—24 IE/mg. Dies war das erste in *kristallisierter* Form dargestellte Proteohormon. SCOTT (188) nahm die Kristallisation des Insulins in Phosphatpufferlosungen der amorphen Hormonpraparate von der Wirksamkeit ca 15 IE/mg in Gegenwart einer geringen Menge verschiedener Metallionen, wie z. B. Zn, Cd, Co oder Ni, vor. Das anfallende Praparat enthalt dann ungefähr 0,5 % Metall, z. B. Zn, und die Ausbeuten an kristallinem Praparat betrugen bis zu 90 %. Kristallisiertes Insulin enthalt zumeist 22—25 IE/mg; LENS (126) beschrieb die Darstellung des reinsten Praparates der Aktivitat 26,8 IE/mg. Aus 5 g Insulin von 24—25 Einheiten erhielt er 3,1 g dieses reinsten Produktes. Außer Acetat- oder Phosphatpufferlosungen zur Kristallisation des Insulins werden neuerdings insbesondere zum Umkristallisieren Citratpuffer (156) angewandt, wobei man gewöhnlich bei pH 6,2 arbeitet Ein Vorzug dieses Verfahrens sind regelmäßige Kristallbildung und hohe Ausbeuten an kristallisiertem Präparat mit der Aktivitat von ca. 24 IE/mg. Die Kristallisation wird in Gegenwart von Zn^{++}-Ionen vorgenommen.

Von den verschiedenen Modifikationen des Isolierungsverfahrens sind namentlich unter Berucksichtigung der erzielten Insulinausbeuten die Unterschiede zu erwahnen, die bei der Extraktion mit angesäuertem Alkohol beobachtet wurden, wenn mit Salzsaure, Schwefelsäure, Essigsäure oder neuerdings mit Phosphorsaure angesauert wurde. Phosphorsaure erwies sich am geeignetsten. In allerletzter Zeit wurden verschiedene Faktoren erortert, welche die Ausbeuten beim Isolationsverfahren beeinflussen. Bereits das verschiedene Behandeln der frischen Drusen beeinflußt die durch Extraktion erzielte Insulinkonzentration (215). Ein vorübergehendes Ansteigen der Aktivitat wird durch Neubildung von Insulin in den β-Zellen der Pankreasinseln erklart, ein Absinken hangt mit der Inaktivierung des Hormons zusammen. Durch verschiedene Abanderungen bei der Hormonextraktion konnen die Ausbeuten an Insulin stark beeinflußt werden, auch wenn das Prinzip der Extraktion mit angesauertem Alkohol beibehalten wird (121) Unter bestimmten Umständen erhalt man durch die Extraktion nur $^1/_{10}$ des vorhandenen Insulins, vielleicht tritt in der ersten Phase der Extraktion eine Verbindung des Insulins mit anderen Substanzen des extrahierten Gewebes ein.

Zur technischen Hormondarstellung dient allgemein ein Verfahren, bei dem die zermahlenen Drusen mit angesauertem Alkohol bei pH 3,0 wiederholt unter Ruhren extrahiert und nach dem Zentrifugieren die vereinigten Extrakte mit Ammoniak auf pH 7,0—7,5 gebracht werden. Der ausgefallene Niederschlag wird abgetrennt,

Insulin wurde bald zu einem unerläßlichen Heilmittel, das aus vielen Tonnen Rinderdrüsen fabrikmäßig im großen erzeugt wurde. Schließlich wurden Präparate mit protrahierter Wirkung ausgearbeitet.

In den LANGERHANSschen Inseln des Pankreas und in Insulinpräparaten wurde noch ein anderer entgegengesetzt wirkender Faktor festgestellt, der Erhöhung des Blutzuckerspiegels bewirkt. Über diesen Faktor lesen wir bereits in der Arbeit MURLINS (146) aus dem Jahre 1923, größere Aufmerksamkeit lenkte er jedoch erst erneut nach dem Jahre 1948 auf sich. Dieser wurde in kristallisiertem Zustand dargestellt, als *Glucagon* bezeichnet und wird als das zweite Hormon der Pankreasinseln betrachtet. Ähnlich wie Insulin besitzt es Eiweißcharakter, und in letzter Zeit wurde seine Struktur durch Aufklärung der kompletten Aminosaurenfolge in seinem Molekül ermittelt. Gegenüber Insulin besitzt es jedoch nur geringe praktische Bedeutung.

Darstellung des Insulins. Das Rohmaterial zur Darstellung dieses Hormons ist Rinder- oder Schweinepankreas, das sofort nach dem Schlachten des Tieres am Schlachthof tiefgefroren wird, um Autolyse durch im Pankreas enthaltene proteolytische Fermente zu vermeiden. Als Notbehelf können die Drüsen anstatt tiefgefroren auch in angesäuerten Alkohol eingelegt oder mit wasserfreiem Natriumsulfat eingesalzen werden (27). Kalbsdrüsen besitzen einen höheren Insulingehalt, sind jedoch fur gewohnlich nicht in größerer Menge zur Verfugung. Zur Insulindarstellung benützte man auch Wal- (28) und Haipankreas (29) und versuchte, das Hormon auch aus den Drüsen von Fischen herzustellen (198, 222), die einen sehr hohen Insulingehalt besitzen. Die gute Qualitat des Rohmaterials ist die erste Bedingung fur eine erfolgreiche Insulinerzeugung zur Erzielung guter Ausbeuten.

BANTING und BEST gemeinsam mit COLLIP (39) extrahierten die zerkleinerten Drüsen mit angesäuertem Alkohol, um die Spaltung des Hormons durch proteolytische Enzyme im Gewebe zu verhüten. Die Extrakte wurden dann nach dem Einengen bei niedriger Temperatur durch Ätherextraktion von den Lipoiden befreit und nach erneuter Anreicherung mit Alkohol konzentriert. Ein Aussalzungsverfahren nach einer ähnlichen Extraktion verwendeten DOISY und Mitarbeiter (70). Es wurden viele verschiedene Modifikationen des Isolationsverfahrens beschrieben, die sich jedoch in der Praxis nicht bewahrten.

Die Grundlage fur die Hormonisolierung bildete das kanadische Verfahren, das BEST ausfuhrlicher beschrieben hat (3, 47).

Die zermahlenen Drüsen werden durch mit HCl angesäuerten Alkohol wiederholt extrahiert, die Gewebsruckstande werden zentrifugiert, der Extrakt mit Ammoniak neutralisiert und der gebildete Niederschlag abgetrennt; die klare Lösung wird nach wiederholtem Ansäuern im Vakuum auf ungefähr $^1/_{15}$ des ursprünglichen Volumens bei einer Temperatur unter 30° C eingeengt, die Lipoidschicht abgetrennt und das Filtrat mit NaCl ausgesalzt. Der Niederschlag wird nun in angesäuertem Wasser

Präparate mit guter protrahierter Wirkung können ferner durch Kombination von Insulin mit verschiedenen Eiweißkörpern erhalten werden. Der Komplex von Insulin mit Protamin, einem aus Fischmilch gewonnenen Eiweiß, erscheint unter der Bezeichnung *Protamin-Zn-Insulin* (*PZI*) sehr häufig in der Praxis. In neutralem wäßrigem Medium ist dieses amorphe Präparat unlöslich; nach der Verabreichung wird das Insulin allmählich aus dem Komplex freigesetzt, wodurch man seine protrahierte Wirkung erzielt. 10 IE Insulin werden mit mindestens 2,5 mg Protamin kombiniert (177). Außer diesen Suspensionen von amorphem Protamin-Zn-Insulin wird neuerdings ein als *NPH* bezeichneter kristallisierter Komplex bereitet, der ebenfalls *eine Suspension darstellt und einen niedrigeren Prozentsatz an Protamin und Zink* enthält. Die Wirkung des Präparates setzt rascher ein, und wie bei dem Präparat *PZI* reicht auch hier zumeist eine Injektion für 24 Stunden aus. Das Präparat wurde erstmals in den Hagedornlaboratorien in Dänemark dargestellt (*N* = neutral, *P* = Protamin, *H* = Hagedorn), und es wird hierfür gute Verträglichkeit angegeben (76). (Tafel. VI/1, neben S. 552).

Ein löslicher Komplex ist *Globin-Zn-Insulin* (*GZI*), das in Lösungen vom pH 3,5 in den Handel kommt. Das Präparat wird durch Bindung von 38 mg aus Hämoglobin bereitetem Protein an 1000 IE Insulin gewonnen (42) und vor allem in Amerika viel verwendet. Der Globintyp des Insulins hat eine ausgeprägt gleichmäßige und langanhaltende Wirkung, die jedoch ebenso wie die des *PZI im Organismus langsamer* einsetzt (161); die Dauer der Wirkung ist etwas kürzer als bei *PZI*. Auch in relativ großen Gaben ruft es nicht so oft nächtliche Hypoglykämie wie *PZI* hervor (118, 171, 190). Ein Nachteil dieser Präparate ist, daß sie eine körperfremde Eiweißkomponente enthalten und so bei verschiedenen Patienten nach einer gewissen Zeit der Verabreichung Überempfindlichkeit bewirken können.

Durch Einwirkung von Phenylisocyanat auf Insulin wurde das sogenannte *Iso-Insulin* mit blockierten Lysin- und Histidin-Aminogruppen dargestellt, das auch protrahierte Wirkung besitzt. Das Gemisch von normalem Insulin und Isoinsulin im Verhältnis 1:1 ist im dänischen Präparat *Di-Insulin* enthalten. Die Wirkung dieses Präparates setzt rasch ein und hält länger an (113, 164); ein ähnliches Präparat unter der Bezeichnung *Kombinsulin* wird in Hoechst erzeugt, und zwar durch Mischung von zwei Teilen Depot-Surfen-Insulin mit einem Teil normalem kristallisiertem Insulin.

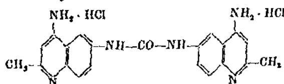
Die *neuesten Insulinpräparate mit protrahierter Wirkung sind kristallisierte oder* amorphe *Hormonsuspensionen* mit einem höheren Zinkgehalt, die ohne Zusatz anderer fremdartiger Substanzen erstmals in Dänemark von der Firma NOVO dargestellt wurden. Das Präparat Insulin Novo *ultralente* ist kristallisiert und besitzt eine protrahierte Wirkung bis zu 36 Stunden, Insulin Novo *semilente* ist der amorphe Typ mit ungefähr 16stündiger Wirkungsdauer. Das Präparat *lente* ist eine Suspension von ungefähr 50% amorphem und 50% kristallisiertem Insulintyp mit protrahierter

das Filtrat erneut angesäuert und im Vakuumverdampfer auf ungefähr $^1/_{15}$ des ursprunglichen Volumens eingeengt. Nach dem Abtrennen der Lipoide erfolgt Aussalzen, und der gewonnene Niederschlag wird zumeist ohne Fällung im isoelektrischen Punkt unmittelbar der Kristallisation und Umkristallisation nach einem der angeführten Verfahren unterworfen. Ein Verfahren fur die fabrikmäßige Erzeugung gemäß der kanadischen Methode beschrieb ROMANS (172) im Jahre 1940, ferner wurden die in der deutschen (69) und dänischen Fabrikation (30) angewandten Verfahren kurz behandelt. Aus Rinder- oder Schweinepankreas kann man über 2000 IE Insulin, berechnet auf 1 kg Drüsenmaterial, herstellen, aus Kalbspankreas über 5000 IE, fur Fischpankreas wurden 25000—40000 IE/kg angegeben; demgegenüber enthält das Walpankreas nur ungefähr 1000 IE Insulin in 1 kg. Zur experimentellen Verwendung wurde auch Insulin aus menschlichem Pankreas dargestellt, und zwar 50 IE auf eine Drüse (84).

Präparate mit protrahierter Wirkung. Normales kristallisiertes Insulin besitzt bei der praktischen Verwendung den Nachteil, daß seine hypoglykämische Wirkung nur kurzfristig ist. Daher beschäftigten sich zahlreiche Versuche mit der Darstellung von Präparaten, die eine länger anhaltende Wirkung aufwiesen.

LINDNER (130) ist es gelungen, durch eine Abanderung des normalen Verfahrens der Insulindarstellung ein Praparat mit protrahierter Wirkung darzustellen, das er als *natives Insulin* bezeichnete. Dieses amorphe Präparat mit 15—20 IE Wirksamkeit wurde aus dem Extrakt durch Fällung nahe dem Neutralpunkt erhalten: es handelt sich offenbar um an ein anderes Eiweiß nativ gebundenes Insulin. Ein ähnliches durch Fällung in Gegenwart von Zink gewonnenes Präparat wurde von BARBIERI (41) dargestellt, es war jedoch weniger wirksam Ebenso verhaltnismäßig schwach wirksam waren Präparate, die durch Komplexbildung von Insulin mit Mucoid und Hyaluronsaure dargestellt wurden (67). Protrahierte hypoglykämische Wirkung wiesen auch die Komplexe von Insulin mit Streptomycin auf (40).

Das sogenannte *Surfen-Insulin* der Firma Bayer in der Bundesrepublik Deutschland ist der Komplex von Insulin mit dem synthetisch hergestellten *bis-2-Methyl-4-aminochinolyl-6-carbamid-hydrochlorid*:



Surfen

Das Praparat weist eine gute protrahierte Wirkung auf, es wurden jedoch auch nach der Präparatzufuhr Nebenreaktionen beschrieben. Intensive verlängerte Wirkung haben Präparate von Insulin mit *Polyvinylpyrrolidon*losung (131).

fest (115). In anderen Fallen wurde empfohlen, die Aktivität des peroral verabreichten Insulins durch verschiedene hypoglykämische Sulfanilamide zu verstarken (132).

Chemische und physikalisch-chemische Eigenschaften des Insulins. (10, 19, 20, 21, 25). Beinahe 20 Jahre seit der erstmaligen Darstellung von kristallisiertem Insulin dauerte die Erforschung der Eigenschaften und chemischen Struktur dieses Hormons an. Als sehr kompliziert erwies sich bereits die Aufklarung des *Molekulargewichts* von Insulin, fur das die Werte 48000, 36000 und 12000 angegeben wurden; bei Verwendung verschiedener Methoden konnte keine Übereinstimmung der gemessenen Werte erzielt werden. Erst später ergab sich, daß die Insulinmolekule unter verschiedenen Bedingungen sich zu Aggregaten *assoziieren* konnen. In 1%igen Hormonlösungen bei pH 6,7—7,5 kommen die Insulinmoleküle zu Aggregaten mit dem Molekulargewicht von ca. 48000 assoziiert vor, während nach Hinzufugen eines Netzmittels oder basischen Eiweißkorpers *Dissoziation* des Komplexes eintritt.

Harfenist und Craig (101) zeigten schließlich, daß die Molmasse des Insulins 5733 beträgt, was als *Minimalwert* angegeben wird (85, 123, 166, 189). Ferner ermittelte man folgende Konstanten:

Sedimentationskonstante	S_{20}	$3{,}55 \cdot 10^{-13}$
Diffusionskonstante	D_{20}	$7{,}53 \cdot 10^{-7}$
isoelektrischer Punkt	pH	5,3—5,4

Bei der Elementaranalyse wurde die folgende Zusammensetzung des Insulins festgestellt:

C	52,96%	N . .	16,04%
H	6,79%	S . .	3,31%

Kristallisierte Präparate enthalten fur gewohnlich 0,3—0,6% Zn (60).

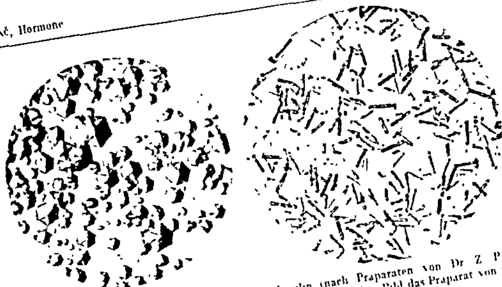
Im Molekul des Insulins wurden nach der Hydrolyse keine anderen Komponenten als Aminosäuren vorgefunden. Mit dem Gedanken, eine „prosthetische Gruppe" zu suchen, die für die physiologische Hormonwirkung im Insulinmolekul verantwortlich ware, beschäftigten sich zahlreiche Arbeiten, namentlich auch die von Freudenberg (86); diese waren jedoch vergebens Das ganze Hormonmolekul besteht aus insgesamt 51 Resten von 16 verschiedenen Aminosauren (worin *6 Cysteinreste* einbezogen sind) Die Gesamtstruktur des Hormons und die Reihenfolge der einzelnen Aminosauren klarte nach mehrjahriger Forschungsarbeit Sanger mit Mitarbeitern (21) durch systematisches Studium der Spaltung des Hormonmoleküls durch Sauren und verschiedene proteolytische Fermente und durch die Analyse der Spaltprodukte auf. Sanger (179) arbeitete eine Methode zur Identifizierung und Bestimmung der endständigen Aminosauren mit der freien α-Aminogruppe mittels *1-Fluor-2,4-dinitrobenzol* aus, die dann, wie bereits an Beispielen anderer Hormone gezeigt wurde, bei ähnlichen Untersuchungen allgemeine Verwendung fand. Bei Insulin wurden als *N-endständige* Aminosäuren *Phenylalanin* und *Glycin* bestimmt

Wirksamkeit. Die Präparate werden durch *Kristallisation* aus Acetatpufferlösung dargestellt. In letzter Zeit wurden die Kristallisationsverfahren sehr gründlich bearbeitet, insbesondere zur Erzielung von Kristallen gleicher Größe und gewünschtem Zinkgehalt (187). Derartige Präparate haben sich in der Praxis sehr gut bewährt, ihr Vorzug ist das *rasche Einsetzen der Wirkung*, die hinreichend lange anhält (62, 81, 98, 107, 133, 174, 186, 196, 206, 214).

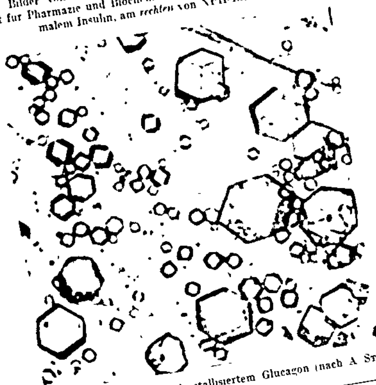
Mit dem Vergleich verschiedener Insulintypen, namentlich von Präparaten mit protrahierter Wirkung, beschäftigten sich zahlreiche Arbeiten (6, 15, 18, 51, 81, 91, 116, 190, 204), die auf die Vor- und Nachteile der einzelnen Präparate hinweisen. Mit den deutschen Präparaten beschäftigt sich ein Übersichtsreferat BAUMANNS (2) aus dem Jahre 1954, besondere Aufmerksamkeit wurde den neuen in Höchst erzeugten Präparaten gewidmet (46, 152, 157). In jüngster Zeit verfolgte man den hemmenden Einfluß von 47 Substanzen, meist synthetischen Derivaten, auf den *Abbau* markierten Insulins. Einige dieser Substanzen weisen hypoglykämische Wirkung auf, und man verfolgt so gleichzeitig ihren Wirkungsmechanismus im Organismus (213). Ferner untersuchte man den Einfluß auf die *verlangsamte Resorption* des Insulins im Organismus, und zwar besonders bei kristallinen Hormonsuspensionen (48) und bei Lösungen mit Harnstoff. Von der Hemmung des Abbaus des Hormons im Organismus und von seiner Resorption hängt eben die protrahierte Wirkung der verschiedenen Präparate ab.

Es fehlt auch nicht an Versuchen, verlängerte Insulinwirkung durch *Implantation* von kristallisiertem Präparat zu erzielen (93, 154). Bei Versuchen an Kaninchen nach subkutaner Implantation gingen infolge Insulinschock innerhalb 24 Stunden 80% der Tiere zugrunde, bei intramuskulärer und intrasplenaler Implantation 40% der Kaninchen. Die größte hypoglykämische Wirkung wurde 4—5 Stunden nach der Implantation beobachtet. Bei Kaninchen mit Alloxandiabetes erzielte man wider Erwarten nur eine kurzfristige Wirkung, da sich um die Peletten bald nach der Implantation eine Hülle bildete, die die weitere Resorption des Insulins verhinderte (93). Obwohl bekannt ist, daß Insulin im Verdauungstrakt inaktiviert wird, wurden Versuche der *peroralen* Hormonzufuhr durchgeführt (170). Mit Hilfe von Pankreaslysaten erzielte man angeblich eine gewisse hypoglykämische Wirkung, so daß zumindest die parenteralen Insulindosen herabgesetzt werden können (64); sonst wurde jedoch nur eine sehr schwache Wirkung auf den Blutzuckerspiegel festgestellt (141).

LASCH (122) empfiehlt zur peroralen Verwendung ultrafiltrabile Insulin-„Subeinheiten", die angeblich von der Schleimhaut des Verdauungstraktes resorbiert werden können; es müssen jedoch antifermentative Substanzen zum Schutz vor den Enzymen zugesetzt werden. Bei Verwendung von Insulindialysaten peroral bei Kaninchen wurde nur eine schwankende Aktivität festgestellt (134). Zur Steigerung der Insulinresorption bei Verabreichung per rectum und per os wurde Hyaluronidase angewandt. Man stellte hinreichende Resorption des Insulins aus der Mundhöhle

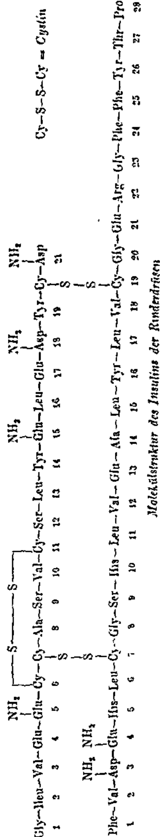


1 Mikroskopische Bilder von kristallisiertem *Insulin* (nach Präparaten von Dr. Z. Pádr, Forschungsinstitut für Pharmazie und Biochemie, Prag). Am *linken* Bild das Präparat von normalem Insulin, am *rechten* von NPH-Insulin



2 Mikroskopisches Bild von kristallisiertem Glucagon (nach A. Staub)

VEB GUSTAV FISCHER VERLAG JENA



Molekülstruktur des Insulins der Rinderdrüsen

Zunächst erwog man, daß bei dem vorausgesetzten Molekulargewicht des Hormons von 12000 (die minimal vorausgesetzte Molekulgröße) das Insulinmolekul aus vier Polypeptidketten besteht; später zeigte sich, daß das Molekul nur *zwei Ketten* enthält. Beide Ketten sind miteinander durch die Disulfidbindung —S—S— der eingegliederten Cysteinreste miteinander verbunden. Mittels Benzopersäure wurden diese Gruppen unter Bildung von $—SO_3H$ oxydiert; dadurch wurden die beiden Ketten aus der gegenseitigen Bindung gelöst.

Man erhielt so zwei Polypeptidfraktionen, von denen die saure mit *A* bezeichnete Fraktion als N-endständige Aminosäure *Glycin*, die basische Fraktion *B* sodann *Phenylalanin* besaß. Allmählich ermittelte man die teilweise Aminosäurensequenz in beiden Polypeptiden vom N-Ende her:

Fraktion *A*: *Glycyl*-isoleucyl-valyl-glutaminsäure,
Fraktion *B*: *Phenylalanyl*-valyl-asparagyl-glutaminsäure.

Weiter ist es gelungen, die völlige Aminosäurensequenz im Polypeptid *A* aufzuklären, das aus 21 Aminosäureresten besteht (182), und die Aminosäurenfolge in der dreißiggliedrigen Kette *B* (181) aufzuklären.

Nur die Lage der Amidogruppen blieb inzwischen noch ungeklärt; wie sich später ergab, gehören von den sechs Gruppen drei der Glutaminsäure und drei der Asparaginsäure an. *Alle drei Reste der Asparaginsäure kommen also im* Insulinmolekül in der Form des Asparagins vor, von den sieben Glutaminsäureresten liegen drei als Glutamin vor und vier besitzen eine freie Carboxylgruppe. Die *C-endständigen* Reste sind in der Kette des Polypeptids A *Asparagin*, beim Polypeptid B *Alanin* (5,87).

Von den sechs Cysteinresten im Insulinmolekül befinden sich vier im Polypeptid A und zwei im Polypeptid B. Sie sind miteinander durch die Disulfidbindung verbunden, und *zwar so, daß die Cysteinreste in Stellung 7 beider Ketten* eine Disulfidbrücke zwischen beiden Ketten und die Reste in Stellung 20_A und 19_B sodann die zweite Disulfidbrücke bilden. Die restlichen zwei in der Kette A in Stellung 6 und 11 bilden durch ihre —S—S—-Bindung einen ähnlichen *Ring*, wie er sich im *Molekul von Oxytocin und* Vasopressin vorfindet (189).

SANGER und Mitarbeitern (21) ist es auf diese Weise gelungen, die Struktur und komplette Aminosäurenfolge des Insulins zu bestimmen. *Es ist dies der erste Fall, daß die Gesamtstruktur eines einheitlichen Eiweißkörpers aufgeklärt wurde.*

Ferner untersuchte man die Eigenschaften der aus dem Pankreas verschiedener Tierarten dargestellten Insulinpräparate. Alle geprüften Präparate waren biologisch gleich wirksam, dennoch wurden gewisse Strukturunterschiede bei Präparaten aus *Rinder-*, *Schweine-*, *Schafs-*, *Pferde-* und *Waldrüsen* ermittelt (55, 102, 180, 185). Eine unterschiedliche Struktur wurde nur in den Stellungen 7—10 der Polypeptidkette A im Molekül von Insulin verschiedener Herkunft ermittelt, die übrige Aminosäurenfolge in dieser Kette sowie die gesamte Struktur der Kette B bleiben dieselbe.

Insulin aus	7	8	9	10
Rinderdrüsen	—Cystin—	Alanin—	Serin—	Valin—
Schweinedrüsen	—Cystin—	Treonin—	Serin—	Isoleucin—
Schafdrüsen	—Cystin—	Alanin—	Glycin—	Valin—
Pferdedrüsen	—Cystin—	Threonin—	Glycin—	Isoleucin—
Waldrüsen	—Cystin—	Threonin—	Serin—	Isoleucin—

Da die verschiedene Aminosäurenfolge zwischen den Cysteinresten 7 und 11 in der Kette A keine quantitativen Veränderungen in der biologischen Insulinaktivität verursacht, wurde gefolgert, daß dieser Teil des Moleküls für die biologische Wirksamkeit des Insulins nicht verantwortlich ist. Genauer läßt sich allerdings nur feststellen, daß der Ersatz von Alanin durch Threonin in Stellung 8_A, von Serin durch Glycin in Stellung 9_A und von Valin durch Isoleucin in Stellung 10_A die Aktivität des gesamten Insulinmoleküls nicht ändert. Die Zusammensetzung des Insulins der Schweine- und Waldrüsen ist völlig identisch.

Es seien nun einige chemische Eigenschaften des Insulins erwähnt. Das Hormon liefert eine Reihe der für Eiweißkörper charakteristischen Farbreaktionen. *Oxydation* des Insulins führt zu seiner irreversiblen Inaktivierung, wie bereits FREUDENBERG im Jahre 1932 und 1935 und nach ihm zahlreiche andere Forscher nachwiesen. Die Oxydation mit Perameisensäure bewirkt Spaltung der die beiden Polypeptidketten verbindenden Disulfidbrücken unter Bildung von HSO_3-Gruppen (179). Dieses Verfahren diente, wie bereits erwähnt, auch zur Strukturforschung des Hormons. Oxydation der phenolischen Hydroxyle der Tyrosinreste mittels Polyphenyloxydase der Kartoffeln bewirkte völlige Inaktivierung des Hormons (112).

Auch *Reduktion* der Disulfidgruppen im Insulinmolekül führt zu seiner Inaktivierung, wie nach Einwirkung von Thioglykolsäure (136) oder Cystein (127) festgestellt wurde. Glutathion wirkt ähnlich, Ascorbinsäure ist jedoch ohne Einfluß. Auf der Reduktion der Cystingruppen des Insulins beruhen die polarographischen Untersuchungen dieses Hormons. Auch Einwirkung von Sulfit bewirkt Reduktion der Disulfidbindungen (59).

infolge Spaltung der Peptidbindungen in beiden Insulinketten ein, man erhält ein Peptidgemisch mit niedrigerem Molekulargewicht und ein größeres cyclisches cystinhaltiges Polypeptid. Die Einwirkung von Trypsin auf Insulin ist nach vorangehender Spaltung mit Chymotrypsin intensiver (58). Durch saure Hydrolyse wird das Hormon allerdings rasch inaktiviert (139). Zur Spaltung des Polypeptids A des Insulinmoleküls dienten auch verschieden aktivierte Pilzproteasen und Papain; aus der Analyse der dabei gewonnenen niedrigeren Peptide konnte ihre äußerst spezifische Wirksamkeit festgestellt werden (184).

Nach der Einwirkung von Carboxypeptidase auf Insulin erzielte man je nach den Bedingungen der Spaltung unterschiedliche Ergebnisse. LENS spaltete so Alanin ab und stellte einen starken Aktivitätsverlust des restlichen Moleküls fest, HARRIS und LI beobachteten unter ähnlichen Bedingungen unveränderte Hormonaktivität. Die Abspaltung von Asparagin führt jedoch zu starker Verminderung der Hormonaktivität, während Abspaltung von Alanin vom C-Ende des Polypeptids B im Insulinmolekül dessen Aktivität nicht beeinflußt (149). Durch saure Hydrolyse des Insulins wurden ein Pentapeptid und ein Heptapeptid gewonnen, die aus dem Polypeptid B des Insulinmoleküls (B 9—13, bzw. B 9—15) herrühren; beim Test mit *Lactobacillus casei* (135) wurde *Strepogenin*-Wirksamkeit festgestellt.

Assoziations- und Dissoziationserscheinungen in Insulinlösungen wurden bereits früher erwähnt. Es wurde auch der Einfluß von Harnstoff, Netzmitteln, schwach basischen Reagenzien, Thiocyanat usw. untersucht. Durch Erhitzen von Insulinlösungen bei pH 2,5 auf 100° C wird das Insulin durch Assoziation in fibrillärer Form gefällt. Diese grundlegende Erscheinung beschrieben bereits in den dreißiger Jahren FREUDENBERG mit Mitarbeitern; WAUGH und FOSTER machten hiervon neuerdings bei der physikalisch-chemischen Bestimmung des Insulins Gebrauch, wie noch gezeigt werden wird. Durch längeres Erhitzen bei pH 1 erhält man Insulin infolge weiterer Assoziation in Form von Sphäriten. Die Reaktionen sind angeblich reversibel und durch Alkalienwirkung bei niedriger Temperatur tritt angeblich wiederum Dissoziation der Aggregate der Insulinmicellen ein.

Eine gewisse Aggregation der Insulineinheiten tritt auch bei der Kristallisation des Insulins in Gegenwart von Zn-Ionen ein. Es wurde die Bindung verschiedener anderer Metalle, wie z. B. von Co, der Einfluß von Fe u. ä.m. verfolgt (68). Auch wurde eine Darstellung von zinkfreien Insulinpräparaten beschrieben (178). Wie bereits erwähnt, führt die Kristallisation von Insulin mit größerem Zinkgehalt zu Präparaten mit verlängerter Wirkungsdauer, offenbar durch Verminderung und Verlangsamung der Resorption derartiger Komplexe. Flüssiges Ammoniak übte bei reinen Insulinpräparaten keinen Einfluß auf deren Eigenschaften aus (74). In allerletzter Zeit wurde festgestellt, daß in den Systemen Pyridinwasser und Essigsäurewasser, die bei der Gegenstromverteilung und der Elektrophorese von Insulinpräparaten verwendet werden, die Moleküle des Insulins dissoziieren (220). Die Lösungsmittel-

Die *Acetylierung* von Insulin bildete ebenfalls den Gegenstand vieler Versuche; zunächst werden die freien Aminogruppen acetyliert, wodurch das Hormon nicht inaktiviert wird, und sodann tritt reversibler Aktivitätsverlust infolge Acetylierung der phenolischen Hydroxylgruppen des Tyrosins ein (62, 195). Zu den Versuchen wurden Keten und Essigsäurechlorid verwendet. Durch *Sulfonierung* des Insulins mit konz. Schwefelsäure bei —18° C erzielt man Blockierung der Hydroxylgruppen der Oxysäuren (168); die gewonnenen Derivate sind biologisch wirksam (94). Mit Metaphosphorsäure bildet das Insulin unlösliche Niederschläge; der Phosphorgehalt entspricht der Zahl der positiv geladenen Gruppen des Insulins (155)

Durch *Veresterung* der freien Carboxylgruppen im Insulinmolekül wird das Hormon inaktiviert; durch Verseifung der gebildeten Ester kann jedoch die Wirksamkeit wiederhergestellt werden (86, 94). Bei Veresterung mit Methanol in saurem Medium tritt jedoch partielle Hydrolyse offenbar der Amidogruppen ein (143). Die Inaktivierung des Insulins durch Diazomethan beschrieb FREUDENBERG im Jahre 1931. Ebenso wurde die Einwirkung von *Formaldehyd* auf Insulin von zahlreichen Autoren mit sehr uneinheitlichen Ergebnissen untersucht. Neuerdings konnte gezeigt werden, daß Formaldehyd bei pH 11—12 nur mit den Amino- und Guanidingruppen des Eiweißkörpers reagiert (83), wobei die Hormonaktivität noch erhaltenblieb. Auch nach Blockierung des Insulins mit 2,-4,-6-Trinitrotoluol (138) wurde Wirksamkeit des Endproduktes festgestellt. Das Ausmaß der Reaktion der freien Aminogruppen bzw. Phenolgruppen hängt hier vom pH des Reaktionsmediums ab.

Bei der *Jodierung* der Tyrosinreste im Insulinmolekül (151) stellten HARINGTON und NEUBERGER reversible Inaktivierung des Hormons fest. LEE (124) markierte Insulin mittels J^{131} und leitet daraus ab, daß bei mäßiger Jodierung die biologische Aktivität des Hormons erhalten werden kann. REINER und Mitarbeiter (167) bereiteten *Azoderivate* des Insulins, und zwar die entsprechende p-Azophenylsulfonsäure und Insulin-p-azobenzyl-trimethylammoniumchlorid in kristallisierter Form, ferner Insulin-p-azojodbenzol und Insulin-p-azophenylarsonsaure. Ferner wurden Absorption und Distribution im Organismus bei Insulin untersucht, das mit diazotiertem mit radioaktivem Jod markiertem Jodanilin gekuppelt war. Durch *Biosynthese* wurde ferner ein mit C^{14} markiertes Insulinpräparat dargestellt, und zwar durch Inkubation von Pankreas mit in Stellung 1 bzw. 3 mit C^{14} markiertem Glycin und Phenylalanin (205). Die Ergebnisse deuten auf den uneinheitlichen Einbau der verwendeten Aminosauren hin, und es wurden daraus Schlußfolgerungen über den stufenweisen Mechanismus der Biosynthese des Hormons und der Eiweißkörper überhaupt aufgestellt.

Viele Versuche wurden auch über die Einwirkung verschiedener Enzyme auf Insulin durchgeführt und der Einfluß auf die Hormonaktivität ermittelt. Diese Versuche trugen auch wesentlich zur Ermittlung der Insulinstruktur bei. Durch Einwirkung von Chymotrypsin tritt verhältnismäßig rasche Inaktivierung des Hormons (159)

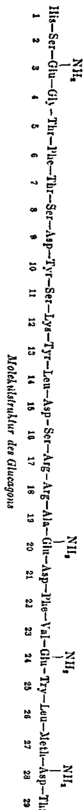
Glucagonzusammensetzung. Es wurde festgestellt, daß das Glucagon ein aus 15 verschiedenen Aminosäuren zusammengesetzter Eiweißkörper mit dem minimalen Molekulargewicht 3482 ist. Bei der stufenweisen Untersuchung der Struktur dieses Eiweißes zeigte sich, daß es aus einer einzigen aus 29 Aminosäureresten zusammengesetzten Polypeptidkette besteht. Als N-endständige Aminosaure konnte nach der Dinitrophenolmethode *Histidin* identifiziert werden; die C-endständige Aminosäure ist *Threonin*. Nach dem bereits bekannten Verfahren der Spaltung des Polypeptids und Analyse der gewonnenen Peptide wurden schließlich auch bei diesem Eiweiß die komplette Aminosäurenfolge und seine Gesamtstruktur aufgeklart (54)

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Gegenüber der ursprünglichen Anschauung uber die Ähnlichkeit der Molekule des Glucagons und Insulins sind nach dem Vergleich beider Strukturen keine Ähnlichkeiten aufzufinden, sei es nun in der Aminosäurenfolge oder der Anordnung der einzelnen Gruppen in den Polypeptidketten beider Hormone oder in den endständigen Gruppen. Von vornherein wurde eine gewisse Übereinstimmung zumindest eines Teils der Molekule beider Hormone vorausgesetzt, und zwar hinsichtlich der Aminosäurenfolge, so wie es bei ACTH und dem Melanophorenhormon zu beobachten war; von verschiedenen Autoren wurde nämlich die Ansicht vertreten, daß die initiale hyperglykamische Reaktion nach Insulinpraparaten auch eine unmittelbare Eigenschaft des Insulins sein kann. Eine derartige Übereinstimmung konnte jedoch nicht ermittelt werden.

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Molekülstruktur des Glucagons

systeme mit Pyridin waren hinsichtlich der Dissoziation aktiver als 6 M-Guanidiniumchlorid und Dimethylformamid und ungefähr so wirksam wie Dimethylacetamid und Trifluoressigsäure.

Darstellung und chemische Eigenschaften des Glucagons (*2a, 9, 17*). In den Mechanismus der Steuerung des Blutzuckerspiegels greift ferner der sogenannte hyperglykämische Faktor des Pankreas (HGF) oder das *Glucagon* ein. Bereits MURLIN beobachtete im Jahre 1923, daß unmittelbar nach i.v. Anwendung von Insulinpräparaten kurzfristige Erhöhung des Blutzuckerspiegels eintritt, worauf dann die spezifische hypoglykämische Wirkung des Insulins folgt (146). Dieser Befund wurde später von zahlreichen weiteren Forschern bestätigt (17). Die für die Erhöhung des Blutzuckerspiegels verantwortliche in den Pankreasextrakten vorausgesetzte Substanz wurde bereits von MURLIN als *Glucagon* bezeichnet.

BÜRGER wies nach, daß nur nicht ganz reine Insulinpräparate eine anfängliche hyperglykämische Wirkung aufweisen, später wurde jedoch festgestellt, daß auch kristallisierte als sehr rein angesehene Insulinpräparate diese Wirkung hatten. Vorübergehende hyperglykämische Wirkung wurde bei allen untersuchten Insulin-Handelspräparaten mit Ausnahme des dänischen Insulins NOVO festgestellt (72). BÜRGER mit Mitarbeiter beschäftigte sich mit Versuchen zur Isolierung von Glucagon aus Insulinpräparaten bereits im Jahre 1935, und es ist ihm gelungen, Fraktionen mit verhältnismäßig starker hyperglykämischer Wirkung herzustellen.

Die Darstellung reiner Glucagonpräparate beschrieb im Jahre 1953 [FOÀ (80)], der von lyophilisiertem Schweinepankreas ausging und die Extraktion mit flüssigem Ammoniak ausführte; Insulin wurde durch Cystein inaktiviert. Der Abdampfrückstand wurde mit physiologischer Lösung extrahiert, und nach dem Einstellen des pH-Wertes auf 7,4 und Abzentrifugieren wurde fraktionierte Fällung vorgenommen. Das gewonnene Präparat war im Test an Kaninchen hochwirksam.

STAUB (193) ging bei der Darstellung von kristallisiertem Glucagon von der amorphen bei der Insulinerzeugung gewonnenen Fraktion aus. Durch Fällung bei pH 6,8 wurde ein Präparat erhalten, das durch Fraktionierung mit Aceton gereinigt wurde. Die bei der Konzentration von 50—56 % Aceton ausgefallene Fraktion wurde sodann in Acetat- und Phosphatpufferlösung und im alkalischen pH-Bereich weiter gereinigt. So erhielt man schließlich ein Produkt von 70 % Reinheitsgrad und nahm daran die Kristallisation vor; Glucagon wurde in Form rhombischer Dodekaeder, die Zinkspuren enthielten, dargestellt. Später wurde jedoch kristallisiertes Glucagon ohne Zink hergestellt, das dieselbe Wirksamkeit aufwies (Tafel VI/2, neben S. 552).

Eine ähnliche Reinigung des Glucagons führten BOSER (49) und ferner DUVE (73) durch. BOSER betrachtete das Glucagon als ein Zuckerkomponenten und Purinbasen enthaltendes Nucleoproteid. Am intensivsten beschäftigten sich die Mitarbeiter A. STAUBS in den Forschungslaboratorien der ELI LILLY Co. in Indianapolis mit der

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His—Ser—Glu(NH_2)—Gly—Thr—Phe—Thr—Ser—Asp—Tyr—Ser—Lys—Tyr—Leu—Asp—Ser—Arg—Arg—Ala—Glu(NH_2)—Asp—Phe—Val—Glu(NH_2)—Try—Leu—Meth—Asp(NH_2)—Thr

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

Molekülstruktur des Glucagons

bei *Mäusen*. Bei dieser zweiten Methode wird der Einfluß der Insulindosis auf die Auslösung hypoglykämischer Krämpfe bei Versuchstieren an größeren Gruppen von Tieren verfolgt. Außer biologischen Testen werden in letzter Zeit zur Bewertung von Insulinpräparaten in Orientierungsversuchen auch physikalisch-chemische Untersuchungen herangezogen, besonders die sogenannte *fibrilläre gravimetrische Methode*.

Die Insulinwirksamkeit wird auf den internationalen Standard bezogen und in internationalen Einheiten (IE) ausgedrückt. Der erste internationale Insulinstandard wurde bereits im Jahre 1925 angenommen und eine Einheit als die Aktivität von 0,125 mg dieses Präparates definiert. Der *zweite* internationale Insulinstandard wurde im Jahre 1935 aufgestellt und enthielt 22 IE in 1 mg des Präparates. Der *dritte* jetzt gültige internationale Insulinstandard enthält in 1 mg 24,5 IE, d. h. 1 IE entspricht 0,04082 mg des Präparates (137).

Der klassische Test für die Bewertung der Insulinpräparate ist die Verfolgung der hypoglykämischen Reaktion nach der Hormonzufuhr *bei Kaninchen*. Man verwendet zumeist Kaninchen, am besten aus einem Wurf und bei Standarddiät; einer Gruppe spritzt man subkutan Standardinsulin von bekannter Aktivität ein und der zweiten das zu untersuchende Insulin. Nach einigen Tagen werden die Gruppen ausgetauscht, so daß man eine Kreuzprobe erzielt und die resistenten Tiere ausschalten kann. Die Blutproben werden ½, 3 und 5 Stunden nach der Hormoninjektion entnommen und die Zuckerbestimmung auf Grund der Reduktionsfähigkeit zumeist nach der Methode gemäß HAGEDORN-JENSEN durchgeführt. Die Senkung des Blutzuckerspiegels in Prozent wird dann für jede einzelne Untersuchung berechnet:

$$S = \frac{I - F}{F} \text{ in } \%,$$

wobei S die perzentuelle Senkung des Blutzuckerspiegels bedeutet, I den ursprünglichen Spiegel und F den nach der Hormonzufuhr gemessenen Blutzuckerspiegel.

Der Test wird von einer ganzen Reihe von Faktoren beeinflußt, namentlich auch von der Wahl der geeigneten Versuchstiere (223). Aus den in 2593 Versuchen ermittelten Werten wurden Korrektionsfaktoren eingeführt, um den Einfluß des unterschiedlichen Anfangsblutzuckerspiegels bei den einzelnen Tieren auszuschalten (108). Es ergab sich eine fast lineare Beziehung zwischen dem Logarithmus der Insulingabe und der erzielten *absoluten* Blutzuckersenkung; in gleicher Weise wurde die Beziehung der *relativen* Senkung des Blutzuckerspiegels gedeutet. Das Verfahren der Insulintitration an Kaninchen wurde oft modifiziert (8), man verwendete verschiedene Applikationen des Hormons, untersuchte den Einfluß wiederholter Dosen, mehrerer verschieden großer Dosen, den Einfluß der Rasse und des Alters der Tiere usw. (s. 22). Die Tiere können in bestimmten Intervallen wiederholt zur Titration herangezogen werden.

Die zweite Methode verwendet größere Gruppen von bei 38° C gehaltenen *Mäusen*, denen das Hormon intraperitoneal verabreicht wird. Die Aktivität des zu prüfenden Insulins wird gegenüber dem Standard nach der Anzahl der Tiere, bei denen hypoglykämische Krämpfe auftraten, statistisch ausgewertet. Die zur Auslösung der Krämpfe, die bei 50 % der Tiere eintraten, erforderliche Dosis betrug nach Hemmingsen und Krogh $^1/_{600}$ der ursprünglichen bei der Bestimmung in Toronto/Kanada verwendeten Kanincheneinheit. Die erwähnten Autoren stellten die Ergebnisse graphisch dar; aus diesen graphischen Darstellungen geht die lineare Abhängigkeit des Prozentsatzes der ausgelösten Konvulsionen vom Logarithmus der angewandten Insulindosis hervor. Auch dieser Test wurde in den nächsten Jahren eingehend bearbeitet und in zahlreichen Einzelheiten modifiziert. So wurden Teste vorgeschlagen, die zwei Dosen des Standards und zwei Dosen des zu prüfenden Hormons verwenden, ferner der Kreuztest u. ä. m. (s. 22). Einen besonderen Einfluß auf die Empfindlichkeit der Mäuse beim Test besitzen die Diät (175), die Temperatur der Umgebung und die Wahl des Tierstammes. Es wurde eine gewisse Abhängigkeit zwischen dem Gewicht des Tieres und der verabreichten Dosis festgestellt, jedoch nicht bei Tieren, die mehrmals in den Testen verwendet wurden (219).

Beim Vergleich beider Titrationsmethoden von Insulin bei Kaninchen und bei Mäusen zeigte sich, daß man bei Verwendung von Präparaten mit sehr unterschiedlichem Reinheitsgrad zu keinen gleichlautenden Ergebnissen gelangt. Die Teste an Mäusen sind weniger mühsam, man muß jedoch viel größere Tiergruppen als bei der Methode mit Kaninchen benutzen. Zumeist verwendet man Gruppen von 10 Kaninchen, im andern Test 50 Mäuse. Zu Orientierungszwecken kann man allerdings auf Kosten der Genauigkeit der Ergebnisse kleinere Tiergruppen verwenden. Young mit Mitarbeitern (217) verglich die mit zwei Modifikationen des Kaninchentests und der Krampfmethode an Mäusen bei verschiedenen Insulin-Handelspräparaten erzielten Ergebnisse und stellte zufriedenstellende Übereinstimmung fest.

Zur Bestimmung von Insulinpräparaten mit Depotwirkung ermittelt man zumeist die ganze glykämische Kurve durch Bestimmung des Blutzuckerspiegels zu verschiedenen Zeitpunkten nach der Hormonzufuhr bei Kaninchen. Eine sehr genaue Methode der Insulinbestimmung schlug Gellhorn mit Mitarbeitern (89) vor, wobei man *hypophysektomierte* Ratten, bei denen auch das Nebennierenmark exstirpiert worden war, verwendet. 0,01 IE Insulin auf je 1 kg Körpergewicht bewirkt bei diesen Tieren bereits hypoglykämische Krämpfe und Coma. Nach dieser Methode ermittelte man den Gehalt von Insulin im menschlichen Blut, und zwar zu 2 IE in 1 l Blut. Zu ähnlichen Testen benutzte man ferner hypophysektomierte und adrenalektomierte Mäuse (77) oder hypophysektomierte Mäuse des Stammes CAF mit Alloxandiabetes (35, 95)

Zur Bestimmung von Insulin im Blutserum oder Plasma verwendete man ferner die Methode, bei der die Utilisation von Glucose an isoliertem *Rattenzwerchfell in vitro*

bestimmt wird. Die Methode ist angeblich so empfindlich, daß sie die Bestimmung der Insulinmenge in 0,32 ml Blutserum ermöglicht (97, 199).

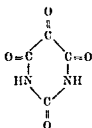
Von den für die Auswertung von Insulinpräparaten verwendeten physikalisch-chemischen Methoden sei vor allem die gravimetrische Bestimmung durch die sogenannte *fibrilläre* Untersuchung angeführt. *Durch Erhitzen der angesäuerten Lösungen* von Insulin entsteht die unlösliche Form des Insulins, wobei sich in der ersten Phase Fibrillen und ein Gel bilden und sodann die einen Niederschlag bildenden Sphäriten auftreten (209) *Diese Aggregation der Insulinmoleküle* wurde bereits in Kürze erwähnt. WAUGH (209) arbeitete auf diesem Prinzip eine Methode zur Insulinbestimmung aus und gibt als Genauigkeit der Ergebnisse 5 % an; für Präparate mit niedrigen Einheiten ist jedoch dieser Test weniger geeignet und viel weniger genau. Nur frisch bereitete Insulinlösungen führen zu richtigen Ergebnissen (82). Das Verfahren ist nicht anwendbar bei Präparaten mit protrahierter Wirkung, wo das Insulin wie z. B. bei Protamin-Zink-Insulin an eine andere Komponente gebunden ist (110). Das fibrilläre Insulin (*F-Insulin*) ist biologisch inaktiv. Bei der Bildung von Fibrillen wirken die Arginingruppen im Insulinmolekül an der Aggregation mit (159).

Hinreichend reine Präparate von kristallinischem und amorphem Insulin konnten durch Elektrophorese (99) nicht unterschieden werden (99); man untersuchte Präparate mit 16—25 IE/mg. LENS (125) beschrieb auf Grund der Untersuchungen der Löslichkeit von Insulin einen Test zur Bestimmung des Reinheitsgrades dieses Hormons Die Bestimmung von inaktiviertem Insulin in Präparaten löste er auf Grund der geringen Loslichkeit in 0,03 N Na_2SO_4 bei pH 3,0. *Auf der Reduktion von Cystin* im Insulinmolekul sind *polarographische* Untersuchungen begründet (202), nach denen eine Bestimmungsmethode vorgeschlagen wurde (145). Im Protamin-Zink-Insulin waren die Cystingruppen polarographisch nicht reduzierbar. *Die polarographische Bestimmung von Zink* im Insulin kann man ohne Mineralisierung der Probe direkt in 4 N Ammoniak vornehmen (162). Die Polarographie diente auch zur Bestimmung des Reinheitsgrades des Insulins (210), ferner zur Identifizierung beim Unterscheiden von gefälschten und unreinen Präparaten (211). Es ist jedoch immer auch die biologische Kontrolle der Praparate fur ihre Wertbestimmung notwendig (92).

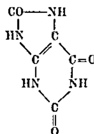
Die Art der Bindung des Zinks in den Insulinpräparaten wurde mittels Titrationskurven der Wasserstoffionen an Präparaten mit verschiedenem Zinkgehalt und ohne Zink untersucht (200). In zahlreichen Arbeiten wurde zur informativen Beurteilung des Reinheitsgrades von Insulinpraparaten das Prinzip der *Papierchromatographie* herangezogen (24, 44, 96, 103, 109, 129, 153, 207), und zwar sowohl in *normaler als auch in ringförmiger Anordnung*; ein Verfahren zur chemischen Bestimmung des Insulins nach Elution und Kjeldahlisierung wurde auch auf Grund der Papierelektrophorese ausgearbeitet (216).

Biologische Eigenschaften des Insulins und Glucagons (3a, 12, 14). Beide Hormone entstehen in den LANGERHANSschen Pankreasinseln. *Insulin* wird von den β-Zellen

dieses Gewebes sezerniert, *Glucagon* von den α-Zellen. Schädigung der Funktion der β-Zellen führt zur Senkung der Insulinbildung, zu Störungen des Kohlenhydratstoffwechsels, die zusammen durch erhöhten Glucosespiegel im Blut zum Ausdruck kommen. J. S. DUNN mit Mitarbeitern beschrieb erstmals im Jahre 1943 die Schädigung der β-Zellen der Pankreasinseln durch *Alloxan* bei gleichzeitiger Auslösung von Hyperglykämie bei den Versuchstieren. Später wurde eine derartige „diabetogene" Wirkung bei zahlreichen andern Substanzen festgestellt. Große Dosen von Harnsäure wirkten bei Kaninchen auch „diabetogen".



Alloxan



Harnsäure

Während Insulin den Blutzuckerspiegel herabsetzt und den Einbau von Glucose zu Glykogen beeinflußt, bewirkt Glucagon hingegen Erhöhung des Blutzuckers durch Anregung der Gluconeogenese aus Glykogen. Aus diesem Grund wird Glucagon oft als *hyperglykämischer* und *glykogenolytischer Faktor* (*HGF*) bezeichnet. Früher wurde Glucagon für den antagonistischen Faktor des Insulins gehalten, neuerdings erscheint jedoch diese Frage durch eine Reihe von Befunden in Zweifel gebracht (31, 34). Glucagon dämpft nicht die periphere Utilisation der Glucose, im normalen Organismus wirken beide Hormonfaktoren des Pankreas bei der Steuerung des Kohlenhydratstoffwechsels derart zusammen, daß sie von einigen Forschern als Synergisten betrachtet werden

Der glykogenolytische Faktor findet sich jedoch auch in den Zellen anderer Organe als nur im Pankreas (78); so wurde er in den abdominalen Lymphknoten und in der Milz nachgewiesen (165), ferner stellte man einen ähnlich wirkenden Faktor im Harn fest (144). Bisher ist nicht geklärt, ob diese Faktoren mit dem von den Zellen der LANGERHANSschen Inseln gebildeten Glucagon identisch sind.

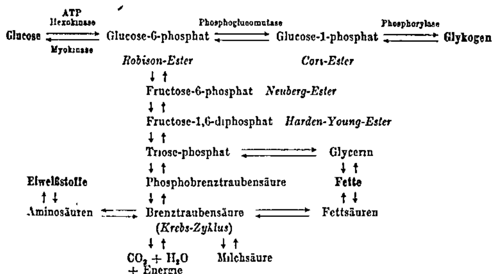
Histochemisch wurde erwiesen, daß nach Zufuhr von Glucagon Atrophie der α-Zellen der Inseln eintritt; hingegen beobachtete man Hypertrophie der β-Zellen (119). Die Herkunft des Glucagons aus den α-Zellen der Inseln konnte man neuerdings bei Versuchen, die Funktion dieser Zellen durch Cobalt(II)-Salze zu hemmen, klar beweisen (43). Mit einem mit J^{131} markierten Glucagonpräparat wurden Versuche durchgeführt, die sein Schicksal im Organismus verfolgen sollten. In der Nierenrinde stellte man ungefähr 5mal höhere Inaktivierung des Glucagons als im Plasma bei

Ratten fest. Leber und Nieren wiesen den stärksten Abbau des Glucagons auf (66, 148). Der Inaktivierungsvorgang ist enzymatischer Natur, er wird von p-Chlormercuribenzoat, Cu^{++}-Ionen, ferner von ACTH und Insulin gehemmt; im letzten Fall handelt es sich um kompetitive Inhibition der Glucagoninaktivierung (111).

In ähnlicher Weise wurde die Inaktivierung des mit J^{131} markierten Insulins bei intakten Mäusen verfolgt (140). Die Inaktivierung ist eine Reaktion erster Ordnung und wird vor allem durch das Enzym *Insulinase* bewirkt. Sehr aktiv in dieser Hinsicht ist hauptsächlich die Leber (140). Beim Hungern nimmt die Aktivität des Enzyms in diesem Gewebe ab. Der größte Teil der zerstörenden Wirksamkeit gegenüber Insulin wurde in den Cytoplasmakomponenten der Leberzellen ermittelt (147). Das inaktivierende proteolytische System der Leberzellen wies nur eine teilweise Spezifität gegenüber Insulin auf; eine gewisse Spaltung stellte man auch bei ACTH, Casein, Glucagon und Somatotropin fest, wenn diese Eiweißkörper als Substrat geprüft wurden (201). Über den Wirkungsmechanismus des Insulins existiert heute eine bereits sehr ausgedehnte Literatur mit oft völlig widersprechenden Ergebnissen, so daß die Orientierung auf diesem Gebiet sehr schwierig ist. In letzter Zeit wurden die Befunde auf diesem Gebiet in einigen übersichtlichen Darstellungen zusammengestellt (11, 12, 13, 16), die auch gleichzeitig eine Diskussion der einzelnen ermittelten Ergebnisse bringen. Eine neue sehr wertvolle Übersicht mit Diskussionen der berufensten Fachleute bietet ferner die im Rahmen der CIBA Found. Coll. Endocrinol. im Jahre 1956 herausgegebene Schrift über die innere Sekretion des Pankreas (26).

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Die Konzentration der Glucose im Blut ist ein wichtiger Faktor für die Versorgung der Gewebe mit diesem energetisch reichen Baustein Die *Leber* spielt hierbei eine bedeutende Rolle, und zwar durch Speicherung oder Spaltung von Glykogen in ihren Zellen. *Hyperglykämie* kann durch hohe Zuckerzufuhr, schlechte Glucoseverwertung, Überproduktion von Kohlenhydraten entstehen, *Hypoglykämie* hingegen bei Entbehrungen, durch übermäßige Verwertung der Zucker, ungenügende Freisetzung der Kohlenhydrate aus den Glykogenreserven. Enzymsysteme katalysieren die einzelnen



Teilreaktionen der Biosynthese oder Spaltung von Glykogen, also auch die Bildung von Glucose und auch ihre weitere Spaltung. In diesen durch eine Reihe verschiedener Faktoren beherrschten Mechanismus greift auch das endokrine System durch Vermittlung der Hormone ein. Außer *Insulin* wurde bereits der Einfluß von *Adrenalin*, den *Rindenhormonen* und dem *Hypophysensomatotropin* auf den Kohlenhydratstoffwechsel im Organismus erwähnt (1, 11, 23). Es ist eine Reihe von Gesamtwirkungen in dieser Hinsicht bekannt, es fehlt jedoch ein näherer Einblick in den Mechanismus der physiologischen Reaktionen in chemischer Hinsicht.

Der Einfluß des Insulins auf den Glucosestoffwechsel wurde namentlich von drei verschiedenen Gesichtspunkten des möglichen Eingreifens verfolgt. Vor allem ist es die Beeinflussung der Biosynthese und der Spaltung des *Glykogens* im Organismus, in der Leber, in den Muskeln und bei Präparaten von isoliertem Zwerchfell in vitro (4); ferner der Einfluß von Insulin auf die *Permeabilität* der Zellmembran gegenüber Glucose und schließlich die Beeinflussung der *Zuckerspaltung*, insbesondere der Oxydations- und Phosphorylierungsprozesse.

Neben der regelmäßigen Senkung des Blutzuckerspiegels nach der Einwirkung von Insulin wird mit Sicherheit Ansteigen des Glykogengehaltes in der Skelettmuskulatur festgestellt. Issekutz mit Mitarbeitern (105_{1951}) ermittelte bei pankreatektomierten Hunden nach Insulinzufuhr während der ersten ½ Stunde erhöhten Zuckerverbrauch im Muskel in situ, der dann unter dem Einfluß des herabgesetzten Blutzuckerspiegels absank. Das Ansteigen des Zuckerverbrauches durch das Muskelgewebe konnte man jedoch durch i.v. Infusion von Zuckerlösung stabilisieren. Auch das Lebergewebe zeigte erhöhten Zuckerverbrauch. Bei intakten Tieren beobachtete man nach Insulinwirkung eine viel höhere Zuckeraufnahme durch das Lebergewebe

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Kohlenstoffanzahl entstehenden Ketonkörper untersucht (53). Bei Insulinmangel stellte man bei Versuchen mit Methionin, das mit radioaktivem Isotop markiert war, gestörte Eingliederung der schwefelhaltigen Aminosäuren in Eiweißkörper, insbesondere in den Nieren, fest (120). Insulin aktiviert angeblich diesen Prozeß.

Das Zentralnervensystem scheint bei der durch Insulin vermittelten Steuerung des Zuckerstoffwechsels nicht beteiligt zu sein (106); man versuchte, einen bedingten Reflex durch Lichtsignale bei Insulinzufuhr bei Versuchstieren hervorzurufen und verfolgte die Reaktion des Blutzuckers, wenn dann nur physiologische Lösung verabreicht wurde. Hierbei trat jedoch keine Senkung des Blutzuckers ein (221). Den ganzen Fragenkomplex über die Entstehung von Diabetes und über die Wirkung des Insulins erörterte neuerdings STADIE (192), der insbesondere die Mitwirkung anderer Hormone, des Somatotropins der Hypophyse und der Nebennierenhormone, hervorhebt. Abnahme der hypoglykämischen Aktivität des Insulins beobachtete man bei in unterkühlter Umgebung gehaltenen hypothermischen Tieren (32). Die bedeutende Erhöhung der Empfindlichkeit der Tiere gegenüber Insulin nach Hypophysektomie und Adrenalektomie wurde bereits bei der biologischen Untersuchung der Insulinpräparate erwähnt.

Nach Einwirkung von Insulin wurde eine interessante Beziehung hinsichtlich der Sekretion des Magensaftes festgestellt (117, 150). Zugleich mit der Beeinflussung des Blutzuckers sank nach Insulin auch die Sekretion des Magensaftes, namentlich auch seine Acidität; die Abnahme wurde später von einem starken Ansteigen abgelöst. Bei Kaninchen stellte man *Antikörperbildung* gegenüber Insulin fest, ähnliche Antikörper werden auch bei insulinresistenten Patienten beobachtet. Es wurde eine auf dieser Grundlage beruhende Methode zur Bestimmung von Insulin in Mikrogrammmengen beschrieben (36). Das Verfahren scheint für die Titration von Insulin in biologischen Flüssigkeiten hinreichend spezifisch zu sein. Auch bei mit einem Präparat von kristallinischem Insulin sensitivierten Meerschweinchen wurde nach i.v. Injektion von Insulin ein anaphylaktischer Schock ausgelöst. Antikörper, welche bei Meerschweinchen oder Schafen Insulin neutralisieren, riefen Resistenz dieser Tiere gegenüber Insulin hervor (142) Die Bildung von Antikörpern gegenüber Insulin wurde bei fünf Tierarten hervorgerufen und bei ihnen die Resistenz gegenüber verschiedenen Insulinmengen verfolgt. Mittels Nachweis durch radioaktive Isotope erfolgte Fraktionierung der Seren von insulinbehandelten Patienten zwecks Lokalisierung der Antikörper, die befähigt sind, Insulin zu binden. In allen untersuchten Fällen war der Antikörper in der Fraktion des α- und β-Globulins vorhanden, nur in fünf Fällen wurde der Antikörper auch in der γ-Globulinfraktion festgestellt (45).

Nun sei noch in Kürze auf das Problem der biologischen Aktivität des Glucagons zurückgekommen. WAELE (208) betrachtet diesen Faktor als ein Hormon, das die Leber vor übermäßiger Lipoidspeicherung schützt. Seiner Ansicht nach besitzt der Faktor sympathomimetische Wirkung, bewirkt bei Katzen Blutdrucksenkung und

als bei Muskelgewebe. STADIE mit Mitarbeiter (191) verfolgte den Einfluß von Insulin auf die Glykogenbiosynthese aus Glucose an Präparaten von isoliertem Rattenzwerchfell. Die Beeinflussung der Glykogengenese sowohl in der Leber als auch im Muskel ist eine der wesentlichsten Insulinwirkungen (90, 105, 218); dieser Prozeß hängt wahrscheinlich mit Phosphorylierungsreaktionen bei der Umwandlung des Zuckers zusammen. Nach Insulinzufuhr beobachtete man Glykogenspeicherung auch in den Fettgeweben (75). Das Hormon hemmt hingegen die Mobilisierung der Glucose aus den Glykogenreserven in der Leber, diese Wirkung des Insulins ist jedoch offenbar indirekt (56).

Insulin erhöht ferner nach den Befunden verschiedener Forschungsstätten die Permeabilität der Zellen verschiedener Gewebe gegenüber Glucose. LEVINE mit Mitarbeiter (16, 128) untersuchte diese Wirkung bei einer ganzen Reihe von Hexosen an verschiedenen Geweben und leitete daraus einige Schlußfolgerungen über die Spezifität des Effektes für ganz bestimmte Zuckerkonfigurationen an den Kohlenstoffatomen 1—3 ab. Die Ergebnisse wurden mit den Befunden über die Utilisation verschiedener Zucker bei der Muskelarbeit verglichen.

Weitere Arbeiten beschäftigten sich mit der Verfolgung der Insulinwirkung auf die Phosphorylierungsprozesse, die mit der Zuckerutilisation im Organismus verbunden sind. CORI und Mitarbeiter (65, 163) stellten die Hemmwirkung einiger aus dem Hypophysenvorderlappen gewonnenen Fraktionen auf die Umwandlung der Glucose in Glucose-6-phosphat fest, die durch Insulin aufgehoben werden kann. Die Phosphorylierungsreaktion wird von dem Enzym *Hexokinase* katalysiert, der Donator der Phosphorsäuregruppe ist dabei die Adenosintriphosphorsäure (ATP). Mittels P^{32} konnte ermittelt werden, daß Einspritzen von Insulin bei Katzen Ansteigen des Verhältnisses der Umwandlung von Phosphokreatin und Adenosintriphosphat während der Glucoseabsorption bewirkt (176). Es wurde auch der Einfluß des Insulins auf die Konzentration des Diphosphothiamins im Blut verfolgt; das Hormon ist offenbar auch bei der Phosphorylierung des Vitamins B_1 beteiligt (79). Außer den Phosphorylierungsvorgängen soll das Insulin auch verschiedene Stufen bei der Oxydation der Zucker beeinflussen (50). Insulin erhöht angeblich auch stark die Oxydation der Glucose zu CO_2, jedoch erst eine bestimmte Zeit nach der Verabfolgung, wie bei der Untersuchung des Stoffwechsels von mit C^{14} markierter Glucose festgestellt wurde (71). Der Zucker verschwindet rasch aus dem Kreislauf, C^{14} ist jedoch weiter vorhanden, woraus man schloß, daß inzwischen eine Reihe von Zwischenprodukten entstehe. Brenztraubensäure wird im Blut diabetischer Tiere nach Glucosezufuhr nicht gespeichert, nach Verabfolgung von Insulin wird jedoch angeblich ihr Gehalt stark erhöht (57).

Katalytische Wirkung des Insulins wurde auch für die Bildung von Oxalessigsäure aus Glucose über Brenztraubensäure vorausgesetzt; diese Frage wurde im Zusammenhang mit der oxydativen Spaltung der beim Abbau von Fettsäuren mit gerader

schen Hepatitiden, ferner auch bei Mastkuren. Das Hormon kommt in den Handel in Injektionsfläschchen zumeist mit Gummiverschluß und einem Inhalt von 5—10 ml Lösung und 100—800 IE Insulin. Die durchschnittliche Tagesdosis beträgt bei der Zuckerkrankheit 10—40 IE je nach dem Blutzuckerspiegel. Langanhaltende Darreichung von exogenem Insulin führt zur Hemmung der Sekretion des eigenen Insulins (61). Durch Verabreichung einer größeren Menge von Kohlenhydraten konnte jedoch diese Dämpfung der Sekretion in einigen Tagen überwunden werden, auch wenn man die Insulinzufuhr fortsetzte.

Wenn bei dem Diabetiker eine viel größere Insulinmenge erforderlich ist, als den Symptomen entspricht, spricht man von *Insulinresistenz*. Diese Erscheinung kann verschiedene Ursachen haben, oft hängt die Resistenzentwicklung mit der Bildung echter Antikörper gegenüber Insulin zusammen (100, 104), in andern Fällen kann es sich um Hyperaktivität der Nebennieren und der Hypophyse (37, 69), ungenügende Absorption der Insulinpräparate oder um übermäßige Spaltung und Ausscheidung des verabreichten Hormons handeln. Nach Darbietung größerer Insulindosen beobachtete man Gesamt- und lokale Komplikationen (212), Störungen des Wasserstoffwechsels im Organismus, Vergrößerung der Leber u. ä. m. Lokale Reaktionen auf

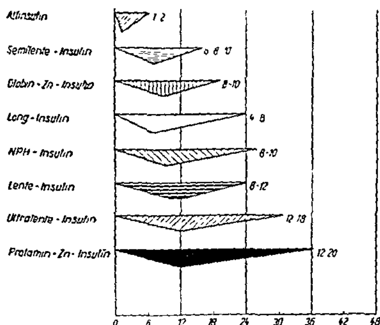


Abb. 9. Schematische Darstellung der Wirkungsdauer verschiedener Insulinpräparate (nach H. K. von Rechenberg Schweiz. med. Wschr., 86, 274, 1956). Auf der x-Achse die Wirkungsdauer in Stunden Der *untere Scheitel* des Dreiecks stellt die Zeit des Wirkungsmaximums bei Angabe der Stunde dar.

bei Meerschweinchen Kontraktion der glatten Muskulatur der Darmwand. Seine Wirkung wird weder von Alloxan noch von Phlorrhizin beeinflußt. Bei niedrigem Glykogengehalt in der Leber kann durch Glucagon keine Hyperglykämie ausgelöst werden (160), ansonsten wurde Steigerung des Blutzuckerspiegels nach Zufuhr des Faktors sowohl bei Normalen als auch bei Diabetikern festgestellt. Zum Unterschied von der Adrenalineinwirkung wurde nach Glucagon keine Beeinflussung des Brenztraubensäuregehaltes im Blute ermittelt (114). Die Anwesenheit von Glucagon in Insulinpräparaten beeinflußt nicht die hypoglykämische Aktivität dieser Präparate, wie durch Krampfteste an Mäusen erwiesen wurde (173). Die glykogenolytische Aktivität des Glucagons ist ungefähr 6mal stärker als bei Adrenalin. Die ausgelöste Hyperglykämie ist bei stabilen Diabetikern ausgeprägter und hält langer an (63). Bei Versuchen zum Studium der Glykogenolyse an Leberschnitten wurde ermittelt, daß Insulin in vitro die Gluconwirkung nicht antagonisiert (203). In jüngster Zeit zeigte sich, daß Glucagon die Ausscheidung von $Na^{\cdot}$-, $K^{\cdot}$-, Cl'- und PO_4'''-Ionen durch die Niere bei anästhesierten und normalen Hunden fördert; der Effekt ist von der hyperglykämisierenden Wirkung abhängig und wird durch direkte Einwirkung auf die Nierentubuli ausgelöst (194)

Infolge der Notwendigkeit, Insulin als Injektion zu verabreichen, wurden Ersatzmittel gesucht, d. h. Hypoglykämie auslösende Substanzen, die peroral verwendet werden könnten. Heute ist bereits eine ganze Reihe von Substanzen mit einer derartigen Wirkung bekannt, die meisten davon versagten jedoch in der Praxis infolge der schwachen Wirkung und beträchtlichen Toxizität. Es wurden verschiedene Guanidinderivate überpruft und das auch als *Synthalin* bezeichnete Decamethylen-diguanidin dargestellt, das eine ausgesprochene hypoglykämische Wirkung besaß, ähnlich das Dodecamethylen-diguanidin, das *Synthalin B* Beachtliche Erfolge erzielte man jedoch in der letzten Zeit mit ganz neuen Arten von Antidiabetica fur perorale Verwendung, nämlich mit n-Butylsulfanilharnstoff (*Nadisan*) und N-(4-Methylbenzolsulfonyl)-N-butylharnstoff (*Rashnon*), die erstmals in Deutschland dargestellt wurden. Der Mechanismus der hypoglykämischen Wirkung dieser synthetisch hergestellten Substanzen ist bisher ziemlich unklar.

Es zeigte sich, daß auch Extrakte verschiedener Pflanzen nach peroraler Darreichung eine allerdings nur verhaltnismäßig schwache hypoglykämische Wirkung besitzen. Hypoglykamisch wirkende Stoffe enthalten z. B. Heidelbeerblätter, Brennnesseln usw. Abnahme des Blutzuckerspiegels wurde durch Glykoside aus den Früchten von *Solanum sanitucongensei* oder das aus den Fruchten der Pflanze *Blighia sapida* isolierte Hypoglycin A bewirkt. Als Antidiabetica fanden auch einige Hefefraktionen und ferner verschiedene Amylasepräparate Verwendung. Pflanzliche Antidiabetica wurden manchmal auch als „*Phytoinsuline*" bezeichnet.

Therapeutische Verwendung des Insulins. In der therapeutischen Praxis verwendet man Insulin vor allem bei der Zuckerkrankheit (*Diabetes mellitus*), bei toxi-

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Insulin wurden bei Diabetikern und bei Normalen nach Zufuhr verschiedener Insulinpräparate untersucht und dabei *festgestellt*, daß sie durch einen *offenbar unspezifischen* Mechanismus *ausgelöst* werden (88).

Günstige Ergebnisse konnten in der Therapie des Diabetes durch verschiedene Präparate mit *Depotwirkung* erzielt werden (s. S. 548). Dank dieser Präparate braucht Insulin nur einmal täglich verabreicht zu werden. Bei akuter Gefahr von Hyperglykämie ist allerdings normales Insulin anzuwenden, dessen Wirkung rasch einsetzt und auch früher abklingt. Durch Einhaltung von Diät wird der Insulinbedarf bei Diabetikern auf ein Minimum eingeschränkt. In neuerer Zeit wurde die Darreichung von Insulin oft durch Anwendung peroraler synthetischer Antidiabetica ersetzt, die *jedoch in manchen Fällen, insbesondere bei jugendlichen Diabetikern und bei bereits lange mit Insulin behandelten Fällen, nicht ansprechen.*

Insulin wird ferner auch bei *psychiatrischen* Patienten zur Auslösung von hypoglykämischem Schock verwendet. Das Auftreten des Schocks hängt eher von der Geschwindigkeit des Absinkens des Blutzuckerspiegels als von dem absoluten Gehalt ab. Durch Glucosezufuhr wird der Patient wiederum auf den Normalzustand gebracht. Bei wiederholter Schockbehandlung erreicht man oft Zustandsbesserung bei Patienten, die an Schizophrenie, Halluzinationen und verschiedenen Wahnvorstellungen leiden Bei Angstzuständen verwendet man manchmal subcomatöse Insulingaben, die nur zur Betäubung des Patienten ohne Verlust des Bewußtseins führen.

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traktion der Drusen nahm er mit 1,5%iger HCl vor. Tweedy (49) fallte die Ballaststoffe mit 60—70% Aceton. Nach Stehenlassen im Kuhlschrank und nach Entfernen des Niederschlags wurde die Losung im Vakuum abgedampft und der Ruckstand mit Trichloressigsaure gefallt. Der Niederschlag wurde mittels Alkohol und Äther weiter gereinigt. Neben der Extraktion mit 3%iger Salzsaure prüfte Tweedy auch die Verwendung von 90%igem waßrigem Phenol. Man erhielt 0,44% Hormon, berechnet auf frische Drüsen, oder 4—5% auf das Trockengewicht der Drusen

Ross und Wood (44) benützten zur Reinigung des Parathormons Aussalzen mit demselben Volumen 2,5 M Ammonsulfat bei pH 5,9—6,0. Der erhaltene Niederschlag wurde nach dem Auflösen in Wasser 3mal unter ähnlichen Bedingungen mit 1,25 M Ammonsulfat ausgesalzt; der Niederschlag wurde dann dialysiert, nach dem Auflosen bei pH 3,5 wurde an Benzoesaure adsorbiert und nach Extraktion mit Äther wurde der unlösliche Ruckstand in verdunnte Saure aufgenommen und dialysiert. Die Darstellung des *Standardpräparates* schlug Dyer (22) bereits im Jahre 1936 aus frischen tiefgefrorenen Rinderdrusen vor, wobei er mittels Pikrinsäure und Aceton reinigte. Das Präparat enthält in 12,5 mg 1 Collip-Einheit.

Auch L'Heureux mit Mitarbeiter (31) benutzte zur Reinigung des Parathormons Fraktionierung mit Aceton, und zwar nach Extraktion der getrockneten entfetteten Drusen mit 0,2 N HCl bei 70—80° C Man erreichte 7—10fache Konzentration der Aktivitat, berechnet auf die im Extrakt vorhandenen stickstoffhaltigen Komponenten. Mit ähnlichem Erfolg wurde die Fraktionierung auch mit Äthanol vorgenommen. Die Wirksamkeit des dargestellten Praparates konnte mit den Präparaten von Ross und Wood verglichen werden; bei der Elektrophorese wurden zwei Komponenten festgestellt. Einige Versuche zur weiteren Reinigung der Präparate von Parathormon, gemeinsam mit der Erprobung verschiedener Methoden zur biologischen Titration der Praparate beschrieb auch Opienska-Blauth (39).

Parathormonpraparate wurden neuerdings auch durch Elektrophorese an Cellulosesaulen gereinigt, nachdem nach dieser Methode verschiedene Eiweißkorper getrennt werden konnten (28), ferner durch Ultrafiltration durch Cellophanmembranen (18). In keinem Fall konnte ein homogenes Präparat dargestellt werden. Zur Isolierung des Hormons aus menschlichem Harn wurden drei Methoden untersucht, und zwar Ultrafilt[illegible] (19). Das letztgenannte Ver-

tat fest, als angeblich bei Eiweißpraparaten gefunden worden war. Bisher wurde jedoch dieser Befund weder von den Autoren ausfuhrlicher beschrieben noch von anderen Arbeitsstatten bestatigt.

Chemische und physikalisch-chemische Eigenschaften des Parathormons. Da das Hormon noch nicht rein dargestellt werden konnte, kann nur eine Übersicht

26. Parathormon

Die Epithelkörperchen wurden von SANDSTROM bereits im Jahre 1880 beschrieben, ihre Bedeutung für den Organismus erkannte man jedoch erst später. Man beobachtete zunächst bei Menschen, dann bei Versuchstieren, daß oft nach Entfernung der Schilddrüse krampfartige tetanieähnliche Zustände auftreten, und zwar in den Fällen, wenn mit der Schilddrüse auch die Epithelkörperchen exstirpiert wurden. Die Epithelkörperchen studierten insbesondere GLEY und ferner KOHN gegen Ende des vorigen und zu Beginn dieses Jahrhunderts.

Besonderes Interesse für diese Drüsen bestand insbesondere von der Zeit an, da McCALLUM und VOEGTLIN (37) die *Beziehung dieser Drüse zum Calciumstoffwechsel* im Organismus erkannten. Den endokrinen Charakter der Epithelkörperchen klärte erst COLLIP mit Mitarbeiter (15) im Jahre 1925 eingehend auf. Durch das Verdienst seiner Schule wurde festgestellt, daß man das für die Drüsenwirkung verantwortliche *Hormon aus Rinderepithelkörperchen darstellen* und durch derartige Präparate bei Tieren, denen diese Drüsen entnommen wurden, Tetanie verhindern oder bei normalen Hunden Anzeichen von Hyperparathyreoidismus auslösen kann. Das Hormon wurde nach den Drüsen *Parathormon* genannt, und später konnte sein Eiweißcharakter festgestellt werden.

Darstellung der Präparate von Parathormon. Am häufigsten verwendet man zur Darstellung des Hormons entweder tiefgefrorene, mit Chloroform konservierte Rinderdrüsen oder das nach Entfetten der Drüsen gewonnene Acetontrockenpulver. Zur Extraktion dienen verdünnte Säuren; im neutralen oder alkalischen Gebiet kann das Hormon nicht extrahiert werden.

COLLIP (15) digerierte frische tiefgefrorene Drüsen mit demselben Volumen 5%iger Salzsäure einige Stunden hindurch im siedenden Wasserbad unter zeitweiligem Umrühren. Nach dem Verdünnen mit Wasser wurde das ausgeschiedene Fett abgetrennt und die Lösung auf pH 8 gebracht. Durch allmähliches Ansäuern wurde das Ballasteiweiß aus dem Extrakt gefällt. Später beschrieb COLLIP eine verbesserte Darstellungs*methode, die zu reineren Hormonpräparaten führt.* Die Fällung der Ballaststoffe wurde wiederholt bei pH 5,0—5,6 vorgenommen. Die Filtrate wurden dann mit NaCl ausgesalzt, der Niederschlag in schwach alkalischer Lösung gelöst und nach dem Zentrifugieren das pH der Lösung auf 4,8 eingestellt, diese Fällung im isoelektrischen Punkt wurde mehrmals *wiederholt, bis die Mutterlauge bereits klar und farblos* blieb. ALLARDYCE (10) benützte zum Fällen des Eiweißes 80% Alkohol bei pH 4,0. Die Ex-

Die ursprüngliche von COLLIP und CLARK (16) im Jahre 1925 veroffentlichte Methode verwendet eine Gruppe von 10 normalen Hunden im Gewicht von ungefähr 20 kg; bei diesen wird der Calciumgehalt im Blutserum bestimmt, das Parathormonpraparat subkutan verabreicht, und nach 15 Stunden wird nochmals das Calcium bestimmt. *Eine* COLLIP*einheit* entspricht $^1/_{100}$ der Praparatmenge, die den Ca -Gehalt im *Blutserum* der Hunde um 5 mg % erhoht. Es wurde kein wesentlicher Unterschied festgestellt, wenn normale oder parathyreoidektomierte Hunde verwendet werden (7), HANSON (30) führt jedoch die zweite Alternative als weniger veranderlich an. Von besonderem Einfluß auf das Ergebnis des Tests ist das Gewicht der angewandten Tiere und ihre individuelle Empfindlichkeit, auch spielt das Alter der benutzten Tiere eine ziemlich große Rolle. Jüngere Tiere reagieren nach COLLIP empfindlicher. Auch ist der ursprüngliche Calciumgehalt im Blutserum zu berucksichtigen; bei einem Grundwert von uber 16 mg % ist die Hormonwirkung bereits wenig ausgeprägt. Ungefahr 5mal kleiner als die COLLIPeinheit ist die HANSON*einheit*, die $^1/_{100}$ der Praparatmenge darstellt, die bei parathyreoidektomierten Hunden innerhalb von 6 Stunden den Ca¨-Gehalt im Serum um 1 mg % erhoht. Dieser Einheit entspricht ungefahr auch *eine USP-Einheit*, die $^1/_{100}$ der Praparatmenge darstellt, die bei 10 Hunden im Gewicht von 8—16 kg nach subkutaner Verabreichung den Ca¨-Gehalt im *Blutserum innerhalb 16—18 Stunden um 1 mg % erhöht*

Im folgenden sind die bei verschiedenen Praparaten ermittelten Aktivitäten, auf 1 mg Stickstoffgehalt umgerechnet, angefuhrt

TWEEDY (49)	55	E/mg N	14,74 % N
COLLIP (15)	*110*	*E/mg N*	*15,5 % N*
ROSS und WOOD (44)	300	E/mg N	12,6—13,1 % N
L'HEUREUX (31)	300		

Das *Titrationsverfahren* nach HAMILTON und SCHWARTZ (29) *mißt den Blutcalciumspiegel* bei Kaninchen, die Methode nach TRUSZKOWSKI und Mitarbeitern (48) bei Ratten; die Bestimmung der Calciumausscheidung bei Ratten liegt dem Verfahren von DYER (21) zugrunde. Die erwahnten Methoden besitzen jedoch nur wenig Vorteile gegenuber der klassischen Methode. BIERING (13) stellte lineare Abhängigkeit fur das Ansteigen des Calciums im Blut bei Ratten vom Logarithmus der Parathormongabe fest und schlug auf diesem Prinzip eine neue biologische Titrationsmethode fur Parathormon vor. Andere Verfahren beruhen auf der Antagonisierung der durch *Magnesiumsulfat bei Mäusen hervorgerufenen Anasthesie durch Steigerung des Calciumgehaltes* im Serum nach Verabfolgung von Parathormon (23)

Zur Bewertung der Parathormonpraparate kann auch die Erhohung der Muskelkontraktionen bei perfundierten Praparaten der hinteren Gliedmaßen beim Frosch *Rana esscutenta* dienen, wenn das zu untersuchende Praparat zur Perfusionsflüssigkeit zugesetzt wurde. Dieses Verfahren untersuchte GELLHORN (27) in seiner Arbeit uber

gegeben werden, welche Eigenschaften der aktiven Komponente bei den nicht homogenen Hormonpräparaten festgestellt wurden. Die dargestellten Präparate lieferten die typischen Reaktionen auf Proteine, auch wird das Hormon durch die üblichen Eiweißfällungsmittel gefallt. Durch saure und alkalische Hydrolyse verlieren die Präparate die biologische Aktivität, auch durch Pepsin und Trypsin werden sie inaktiviert. Das Hormon scheint keine eiweißfreie prosthetische Gruppe zu enthalten, die durch ultraviolette Spektrographie der Praparate festgestellt werden könnte.

Ross und Wood (44) stellten nach dem Verhalten der Hormonpräparate in der Ultrazentrifuge zwei Komponenten mit der Molmasse 500000 bis 1000000 und 15000 bis 25000 fest. Der zweite Bestandteil bildet ungefähr 65 % des Präparates und hatte über 50 % der Gesamtwirkung des Präparates. Im Trockenzustand ist das Hormon sehr beständig, ebenfalls in schwach sauren Lösungen vom pH 3—5; in neutralen und alkalischen Lösungen verliert das Hormon langsam die Aktivität. 5 %ige NaOH bewirkt völlige Inaktivierung des Hormons. Die Angaben über den isoelektrischen Punkt des Hormons gehen ziemlich auseinander. Collip gibt den Wert 4,8 an, was Allardyce bestätigt, wenn vom sauren pH-Bereich ausgegangen wird. Wird hingegen das pH vom alkalischen Bereich herabgesetzt, so tritt die Fällung bereits bei pH 6,0 ein. Tweedy und Mitarbeiter (49) geben als isoelektrischen Punkt des Parathormons den Wert 5,8 an Die wirksamsten Praparate von Ross und Wood wurden bei pH 4,5—5,0 gefällt.

Das Hormon ist löslich in Wasser, in schwachen Salzlosungen, in schwacherem wäßrigem Alkohol, in Essigsäure, in der Wärme in Phenol und Glycerin. Unlöslich ist es in absolutem Alkohol, in Äther und anderen organischen Losungsmitteln. Parathormon wird durch Formaldehyd, salpetrige Säure, Wasserstoffperoxyd, Kaliumpermanganatlösung oder saure Alkohollosungen inaktiviert. Tweedy beschrieb in einigen Fällen teilweise Wiederherstellung der Hormonaktivität. Schwefelwasserstoff oder allgemein Reduktions- und Hydrierungsmittel bewirken keine Inaktivierung des Hormons. Keten stört die Hormonaktivität irreversibel

Zum Unterschied von andern Proteohormonen führten Versuche zur Trennung der Komponenten des Parathormonpraparates durch Gegenstromverteilung, Verteilungschromatographie oder Adsorptionsverfahren nicht zum Ziel (Handler, s 3_{1955}). Auch Trennen der Komponenten mittels des Ionenaustauschers Dowex-50 durch Gradient-Elutionstechnik im pH-Gebiet 2,8—11,0 bewirkte keine Anreicherung der Aktivität in einer Komponente, alle Komponenten besaßen ähnliche Wirksamkeit.

Biologische Bewertung der Parathormonpräparate (8). Zur biologischen Titration dieses Hormons werden vor allem zwei Gruppen von Methoden benützt. Die erste beruht auf der Bestimmung der Steigerung des *Calciumgehaltes* im Blut nach der Hormonzufuhr, die zweite auf der Verfolgung der Senkung des *Phosphorgehaltes* im Blut.

Sehr eingehend wurde insbesondere die Beziehung des Parathormons zum Knochenstoffwechsel untersucht (24), zu Vitamin D und Dihydrotachysterin (AT 10), das allerdings nach einem ganz andern Mechanismus den Ca··-Metabolismus ebenfalls bedeutsam beeinflußt. Die Wirkung länger anhaltender Hormonzufuhr auf die Chemie der Knochen wurde bei jungen Hunden mit verschiedener Ca-Zufuhr (14) untersucht; bei Meerschweinchen verfolgte man nach Anwendung des Hormons insbesondere die Histologie der Knochen und Zähne (34). Während Vitamin D_2 die Osteogenese fördert, namentlich nach Zufuhr von Glycerinphosphat und Calcium, regt das Parathormon hingegen die Demineralisierung der Knochen an, erhöht den Calciumspiegel im Blut sowie die Calciumausscheidung durch die Nieren und auch auf dem Darmwege. Das Parathormon reguliert den Ca··-Blutspiegel ähnlich wie Insulin den Zuckerspiegel Die Calciumreserve bilden hier die Knochen. Neuerdings wurde festgestellt, daß Vitamin D die Wirksamkeit des Parathormons verlängert und das Hormon wiederum die Hemmwirkung des Vitamins auf die Phosphorresorption in den Nierentubuli erhöht (33).

Die Ausschüttung des Hormons durch die Epithelkörperchen richtet sich nach der Höhe des Calciumspiegels im Blut (3); die hypercalcämische Aktivität des Hormons hängt nicht nur von der Nierenfunktion ab, obwohl das Hormon die Reabsorption der Phosphate in den Nieren herabsetzt (33, 36, 41). Ähnlich wie die Ca··-Ausscheidung durch Parathormonzufuhr erhöht wurde, konnte ein ähnlicher Effekt auch für Sr·· festgestellt werden (11). Die Fraktionen verschieden gereinigter Hormonpräparate mobilisieren das Ca·· mit verschiedener Geschwindigkeit. Bei reineren Präparaten setzt die Wirkung rascher ein, klingt aber auch rascher ab (42).

Beim Studium des Wirkungsmechanismus von Parathormon*) ergab sich, daß das Hormon offenbar die organische Knochenstruktur unmittelbar beeinflußt, wodurch z B bei Hyperfunktion der Epithelkörperchen Demineralisierung eintritt (40). Das Parathormon greift hier in den enzymatischen Mechanismus der Citratutilisation im Knochengewebe ein, d. h. die Isocitronensäuredehydrogenase bzw. Coenzym II. Das Parathormon zerstört die chromophore Gruppe der reduzierten Coenzymform, wie V [illegible]
n [illegible]
s [illegible]
gradienten könnte man auch die Mobilisierung der Ca··-Ionen aus den Knochen erklären. Neuerdings wurde der Einfluß der Nephrektomie auf den Gehalt an Ca·· und Citronensäure im Blut in Verbindung mit der Funktion der Epithelkörperchen ermittelt (25). Bei Kaninchen und Katzen verfolgte man in allerletzter Zeit den Einfluß des Parathormons auf den Einbau von P^{32} in die endoosteale Schicht der Diaphyse (20) Sowohl bei normalen als auch bei nephrektomierten Individuen beider Tierarten tritt Abnahme der P^{32}-Aufnahme ein.

*) Siehe auch G. R. Martin J. amer chem Soc. 80, 6201, 1958

den Einfluß von Parathormon *auf den Muskel im hypodynamischen Zustand*; die Methode wurde jedoch nicht als Verfahren zur Bewertung des Hormons ausgearbeitet. THORP (8) ist der Meinung, daß sich dieses Prinzip als schnelle Titrationsmethode *für Parathormon verwerten ließe.*

Eine verhältnismäßig einfache und sehr empfindliche Methode ist die Bestimmung der Abnahme des Phosphors im Serum bei erwachsenen Ratten nach Verabreichung von Parathormon, wie sie von TEPPERMAN und Mitarbeiter (47) im Jahre 1947 ausgearbeitet wurde. Dieser Autor stellte fest, daß die Abnahme des Gehalts an anorganischem Phosphor nach subkutaner Hormonzufuhr dem Logarithmus der Dosis des verabreichten Praparates direkt proportional ist. Die Tiere müssen bei einer bestimmten Diät gehalten werden, denn bei hungernden Ratten wurden Fehlergebnisse in den ermittelten Aktivitäten *der geprüften Präparate festgestellt.*

Das Verfahren RUBINS und DORFMANS (45) beruht auf der Bestimmung der P^{32}-Ausscheidung durch den Harn bei Ratten, denen gleichzeitig das Parathormonpräparat und 1,2 mg mit P^{32} markiertes Na_2HPO_4, *berechnet auf 100g Körpergewicht,* verabreicht wurden. Die Injektion wird intraperitoneal verabfolgt, und die Bestimmung erfolgt 24 Stunden nach der Parathyreoidektomie der Versuchstiere. KENNY und Mitarbeiter (35) bestimmten *die Gesamtausscheidung von P bei Ratten innerhalb* 6 Stunden, und zwar unmittelbar nach der Parathyreoidektomie und der Darbietung der Parathormonpraparate. Die Empfindlichkeit des Tests war jedoch geringer als beim vorangehenden Test.

Erwähnenswert sind noch Versuche zur Verfolgung des Citronensäuregehaltes im Serum von parathyreoidektomierten Ratten nach der Zufuhr von Parathormonpräparaten (32). Zu den Versuchen verwendete man junge geschlechtsreife weibliche Ratten, denen 24 Stunden nach der Entfernung der Schilddruse und der Epithelkörperchen eine bestimmte Menge Parathormon subkutan verabreicht wurde; nach 18 Stunden stellte man die Erhohung des Citronensäuregehaltes im Serum fest. Auch dieser Effekt kann die Grundlage für die Titration von Parathormonpraparaten bilden.

DAVIES und GORDON (17) verglichen die auf der Bestimmung von Calcium und Phosphor beruhenden Titrationsverfahren, insbesondere bei der Verwendung parathyreoidektomierter Ratten, und fuhrten zahlreiche Modifikationen der Methoden an.

Biologische Eigenschaften des Parathormons (1, 2, 3, 4, 5, 6, 7). Die Hormonwirkung bei parathyreoidektomierten Tieren kommt vor allem in der raschen Normalisierung des Gesamtzustandes *zum Ausdruck, vor allem dadurch,* daß keine Tetanien auftraten, denen die operierten Tiere nach Abnahme des Calciumgehaltes im Blut und bei erhohter Nervenreizbarkeit sonst unterliegen. Die Steigerung des Calciumspiegels im Blut und Abnahme des Phosphors nach Verabreichung von Parathormon tritt nicht nur bei parathyreoidektomierten, sondern auch bei normalen Tieren auf.

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Ferner wurden einige Wirkungen der Epithelkörperchen beschrieben, die nicht unmittelbar mit dem Ca^{++}- und Phosphorstoffwechsel im Organismus zusammenhängen. Diese sind nicht immer mit Bestimmtheit dem Parathormon zuzuschreiben, denn auch wenn man mit Hormonextrakten und -konzentraten arbeitet, können diese Wirkungen von anderen Substanzen ausgelöst werden. Einige können sekundär hervorgerufen werden, manche sind möglicherweise Artefakte (3).

Nach der Verabfolgung von Extrakten der Epithelkörperchen, die hypercalcämisch wirksam waren, beobachtete man bei Ratten vermehrtes Serumglykoprotein und Seromucoid. Nach dreitägiger Verabreichung wurden Läsionen in den Nieren hervorgerufen und Calciumablagerung festgestellt; durch Farbreaktionen wurden auch Polysaccharide ermittelt (46). Bei normalen Ratten bewirkt der parathyreoide Extrakt erhöhte Ausscheidung des Mucoproteins durch den Harn (26). Parathormon wirkt weder auf den Gehalt an Eiweiß noch an Chloriden im Blut. Es beeinflußt weder den Grundumsatz noch den Blutdruck.

Parathormonpräparate sind in der ärztlichen Praxis nicht sehr geläufig. Sie kommen bei Tetanien, bei Eklampsie und leichten Lungenhämorrhagien, im allgemeinen jedoch recht selten zur Anwendung. Das Hormon erscheint praktisch in Injektionen mit 20 CE, die ähnlich wie Insulinpräparate hergestellt werden (9). Neuerdings wurde über die *Behandlung von akuter Nierenischämie mit Parathormon in Infusionen* berichtet. Bei allen so behandelten Patienten kamen Anurie oder Oligurie bald zum Verschwinden, wahrscheinlich durch Beeinflussung der Nierentubuli, deren Funktion weiterhin geschädigt bleiben kann (12).

Von den Handelspräparaten des Parathormons seien angeführt:

PARATYRONE	Lab. Byla Paris Frankreich
PARATHYROIDE	G. Richter, Ungarn
PARA-THOR-MONE	Eli Lilly Co. USA
PARATHYROID EXTRACT	Armour USA
PARATHYROID GLAND	Burroughs Wellcome England
PARATYREOIDIN	Moskauer Werke UdSSR
PAROIDIN	Parke Davis USA

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gestellt. HISAW (20) ähnlich wie FEVOLD (12) extrahierten die Organe mit Alkohol, der mit 2% HCl angesäuert war; nach dem Neutralisieren und dem Verdampfen des Extraktes wurde das Hormon durch Fraktionierung mit Äthanol und Äther weiter gereinigt. ABRAMOWITZ (5) und ALBERT mit Mitarbeiter (6) extrahierten die getrockneten entfetteten Gelbkörper mit 2% Salzsaure und reinigten das Hormon durch fraktioniertes Aussalzen mit Ammonsulfat und Fallung mittels Äthanol. Die Ausbeuten aus den ganzen Ovarien waren vielhundertfach höher als aus den Gelbkörpern allein. Die Ovarien trächtiger Saue enthielten 500—1500mal mehr Relaxin als die Ovarien der normalen Tiere (7).

FRIEDEN und HISAW (13) beschrieben ein Verfahren zur Reinigung von Relaxin, wobei sie die homogenisierten Ovarien trächtiger Saue mit 2% HCl oder 1 M Natriumacetatlösung extrahierten und den Rohextrakt durch Fallung und mehrmals wiederholte Dialyse reinigten; das Praparat enthielt nach der Gefriertrocknung 30—75 Meerschweincheneinheiten in 1 mg. In einem andern Verfahren (1) ging man entweder von Homogenaten der Ovarien oder von Acetontrockenpulver aus; dies wurde wiederholt mit 5%iger Salzsäure extrahiert und der Extrakt nach dem Zentrifugieren von begleitenden Ballaststoffen durch Fällung mit Alkohol bis zu 42% befreit. Das Filtrat wurde nach dem Zentrifugieren mittels Essigsäure auf pH 4 eingestellt, und nach Abtrennen des Niederschlages wurde das Filtrat mit Äthanol bis zur Konzentration 80% gefallt. Vom entstandenen Niederschlag wurde abfiltriert und das pH des Filtrats mit NaOH auf 6,5 gebracht, sodann wurde der Niederschlag abgetrennt und die Losung nach dem Zentrifugieren mit dem 4fachen Volumen Aceton gefallt. Darauf wurde der Niederschlag nach Auflosen in Wasser zum Entfernen der Elektrolyte dialysiert und das Praparat schließlich lyophilisiert Das Präparat enthielt 30 bis 100 Meerschweincheneinheiten in 1 mg, und die Ausbeute betrug ungefahr 1,1—1,8 g Präparat auf 1 kg frische Drüsen

Durch weitere Reinigung mittels Fraktionierung mit Aceton bei pH 6,5 kann man zu einem Präparat mit 300—450 Meerschweincheneinheiten in 1 mg gelangen, wobei die Ausbeute ungefahr 60% der gesamten ursprunglichen Einheiten beträgt (1) Die Reinigung von Relaxinpraparaten durch Chromatographie an Amberlit IRA-400 beschrieb neuerdings LEHRMAN mit Mitarbeitern (21). Außer dem erwahnten Anionenaustauscher benutzte man auch Amberlit IR-120, wodurch der Großteil der Ballaststoffe und Polypeptide mit freien basischen und sauren Gruppen beseitigt wurde Relaxin wird an derartigen Saulen nicht festgehalten Die Aktivitat der Eluate war nach Umrechnen auf den Gesamtstickstoff ungefahr 10mal hoher. Weitere Fraktionierung wurde an oxydierter Cellulose und an dem Kunstharz Dowex-50 gepruft (14). Im ersten Fall erhohte sich die Reinheit der Praparate 2—3mal bei einer ungefahr 60%igen Ausbeute an der ursprunglichen Gesamtaktivitat, im zweiten Fall wurde 6—8fache Reinigung bei einem Verlust von 50—70% der ursprunglichen Gesamtaktivitat erzielt.

27. Relaxin

Die einzelnen Faktoren für die Lockerung der Beckenbänder fur die bevorstehende Geburt wurden bei Frauen bereits im vergangenen Jahrhundert untersucht; das Relaxationsphanomen bei Meerschweinchen wurde bereits im Jahre 1812 von LE GALOIS erkannt. Im Jahre 1926 erwog HISAW (19) die endokrinen Einflüsse auf die Relaxationsreaktion bei Meerschweinchen. Das betreffende Hormon, das die Lockerung der Beckenbänder einleitet, wurde später *Relaxin* genannt. HISAW konnte dann in späteren Arbeiten nachweisen, daß das Hormon im Blutserum gravider Kaninchen, Hunde, Katzen, Meerschweinchen und Stuten enthalten ist und daß für seine Wirksamkeit *unbedingt notwendig ist, auf den Organismus zunächst Östrogen einwirken* zu lassen. Diese Schlußfolgerung ergab sich aus an kastrierten Meerschweinchen durchgefuhrten Versuchen, wobei den Versuchstieren relaxinhaltige Präparate verabfolgt wurden.

In den dreißiger Jahren wurde von verschiedenen Forschern festgestellt, daß bei kastrierten Meerschweinchen Östrogen an sich oder in Kombination mit Progesteron auch eine gewisse Lockerung der Beckenbänder hervorruft. Man begann also die Existenz des Relaxins zu bezweifeln. Als wirksam erwiesen sich *insbesondere Gelbkörperextrakte*. BROUHA und DESCLIN untersuchten die Relaxationsaktivität wäßriger und lipoider Gelbkorperextrakte von Säuen an vorher mit Östrogen vorbereiteten Meerschweinchen. *Die wäßrigen Extrakte waren voll wirksam*, während die Lipoidextrakte wirkungslos waren. Die durch Östrogen allein ausgelosten Relaxationen setzten nach ungefähr zweimal so langer Zeit nach der Verabfolgung ein, als wenn wäßrige Gelbkorperextrakte verwendet wurden. Diese Versuche zur Auslosung der Relaxation durch Steroidhormone wurden spater an verschiedenen Stellen mit ähnlichen Ergebnissen oft wiederholt.

In den Gelbkorpern existiert also neben Progesteron noch ein weiteres Hormon- *Relaxin —, das wasserloslich ist und Polypeptidstruktur besitzt.* Das Hormon konnte außerdem auch im Stroma der Ovarien, nicht jedoch im Follikelliquor nachgewiesen werden, in geringer Menge wurde es auch in Uterus und Plazenta gefunden. Wahrend des Sexualzyklus tritt es nur in der Luteinisierungsphase auf, und zwar im Vergleich zu der Menge wahrend der Graviditat in verhältnismaßig geringer Menge. Relaxin ist ein Schwangerschaftshormon (2).

Darstellung von Relaxinpräparaten. Das Hormon wird aus Gelbkorpern von Schweinen oder besser aus ganzen Ovarien zumeist durch saure Extraktion dar-

ohne daß diese durch Östrogendosen zum Stillstand gebracht werden kann Vielleicht liegt Bildung spezifischer Antikorper gegenuber Relaxin vor.

Ein weiteres Verfahren der Auswertung von Relaxin an Mausen arbeitete Hall (16, 17) aus, wobei die Relaxationen des Beckens röntgenographisch festgehalten werden. Mäusen werden täglich 1,5 μg Östron 8 Tage hindurch verabfolgt; sodann wird 24 Stunden nach einer einzigen Injektion des Relaxinpraparates die Untersuchung vorgenommen und mit dem 24 Stunden vor der Injektion des zu untersuchenden Präparates ermittelten Befund verglichen. Neuerdings wurde die radiographische Methode als empfindlich und praktisch gut verwendbar befunden (9). Hierbei werden die Tiere nur einmal fur das Austesten verwendet. Die röntgenographische Bewertung der Relaxation kam später auch bei Meerschweinchen zur Anwendung (s. 2).

Biologische Eigenschaften des Relaxins. Wie bereits erwahnt, wird das Hormon vor allem in den Gelbkörpern der Ovarien gebildet; diese Wirksamkeit wurde jedoch auch im Stroma der Ovarien, im Uterus, in der Plazenta und im Blutserum trächtiger Kaninchen gefunden. Es hat den Anschein, daß der Relaxationsfaktor auch im Kaninchenuterus entstehen kann Im Serum von graviden Kaninchen wurden normalerweise 10 Meerschweincheneinheiten auf 1 ml festgestellt. Bei Meerschweinchen ermittelte man am 21. Tag der Gravidität 0,25 Meerschweincheneinheiten in 1 ml, am 28. Tag 0,5 Meerschweincheneinheiten Relaxin im Serum. Zwischen dem 28. und 56. Tag der Gravidität wurden durch den Harn 5—10 Meerschweincheneinheiten ausgeschieden Im Uterus wurden am 56. Tag der Gravidität ungefahr 10 Meerschweincheneinheiten auf 1 g frisches Gewebe, in der Plazenta 5 Einheiten gefunden (28).

Relaxin bewirkte nach der Vorbereitung der Tiere mit Östrogen bei kastrierten Mausen in den Symphysen des Beckens ein ahnliches histologisches Bild, wie wir es bei der Graviditat vorfinden (16). Es wurde festgestellt, daß das Relaxin von der Einstichstelle aus rasch absorbiert wird und auch auffallend rasch aus dem Blute verschwindet. Ungefahr 50% der verabfolgten Menge verschwindet innerhalb einer Stunde nach der Injektion, der Rest dann innerhalb 24 Stunden. Maximale Hormonkonzentration im Blute wurde 5 Minuten nach subkutaner Verabfolgung ermittelt. Relaxin wird offenbar rasch inaktiviert, nur ein geringer Anteil der Aktivität der verabreichten Hormonmenge konnte im Harn bei Kaninchen gefunden werden (26) Die antigene Aktivität des Relaxins ist offenbar nur schwach.

Relaxin aus den Ovarien trächtiger Säue und auch aus dem Blut trachtiger Kaninchen wirkt antidiuretisch (27), es beeinflußt gewissermaßen in Kombination mit Östradiol und Progesteron das lobulo-alveolare Wachstum der Brustdruse bei ovariektomierten Ratten (11, 24). Das Hormon bewirkt die Bildung oder Aktivierung des „Spreading"-Faktors in den Milchdrusen Ferner prufte man auch die Kombination mit Luteotropin auf diesen Effekt

Chemische und physikalisch-chemische Eigenschaften des Relaxins. Das Hormon ist ein Polypeptid mit der Molmasse 10000—12000, wasserlöslich, sein isoelektrischer Punkt liegt ungefähr bei pH 7,0, der Stickstoffgehalt beträgt bei vorläufig nicht ganz homogenen Präparaten 13,9—14,8% (1).

KRAINTZ (s. 1) stellte bei der Analyse der Aminosauren des Hormons nach sauerer Hydrolyse mit Hilfe von Papierchromatographie die folgenden Komponenten fest: *Glycin, Alanin, Serin, Valin, Lysin, Cystin oder Cystein, Glutamin-* und *Asparaginsäure, Tyrosin und Histidin.* Die reinsten Hormonpräparate enthielten nur Spuren von Hexosen und Hexosamin. Aus dem abweichenden Verhalten verschieden dargestellter Präparate bei der Elektrophorese wurde geschlossen, daß das Relaxin in Rohpräparaten wahrscheinlich an Eiweiß gebunden ist, es ist allerdings nicht ausgeschlossen, daß einige Molekülformen mit Relaxinwirksamkeit bestehen.

In neutralen Lösungen ist das Hormon relativ thermostabil, durch Temperaturerhöhung bei sauren oder alkalischen Lösungen wird jedoch die Aktivität rasch zerstört. Auch Reduktionsmittel und proteolytische Enzyme inaktivieren das Hormon rasch. Durch Einwirkung von salpetriger Saure oder durch Acetylierung wurde die Hormonaktivität nicht wesentlich beeinflußt, durch Methylierung wird sie jedoch rasch zerstört (1). Nach LEHRMAN enthält das Hormon keine freien basischen oder sauren Gruppen, wie sich aus dem Verhalten an mit Ionenaustauschern beschickten Säulen ergab.

Biologische Bewertung von Relaxinpräparaten. Die Hormonbestimmung erfolgt durch Ermittlung der Relaxation des Bindegewebes im Becken zumeist bei Meerschweinchen, und zwar durch Palpation oder mittels Rtg-Strahlen nach der Verabfolgung des Hormonpraparates.

Das grundlegende Verfahren der Auswertung arbeitete HISAW (18) im Jahre 1927 zur qualitativen Bewertung der Praparate aus. Später definierte man die Einheit der Relaxinaktivitat als die minimale Hormonmenge, die binnen 10—12 Stunden nach einer einzigen Injektion des Praparates eine gewisse Lockerung hervorruft. ABRAMOWITZ untersuchte ferner die Bedingungen bei diesem Test und veroffentlichte die Standardkurve der Abhangigkeit der biologischen Reaktion von der Hormongabe, welche eine typische sigmoide Form besitzt. Als eine Meerschweincheneinheit der Wirksamkeit des Praparates wurde sodann diejenige Hormonmenge definiert, die Relaxation der Symphysen des Beckens bei 8 von 12 kastrierten Meerschweinchen im Gewicht von 350—800 g bewirkt. Die Tiere erhielten vorher insgesamt 4 Tage hindurch täglich 0,83 μg Östradiol. Palpation der Symphysen des Beckens wird 6 Stunden nach der Einspritzung des Relaxinpraparates vorgenommen (s. 2). In allerletzter Zeit wurden die Veränderungen in der Empfindlichkeit der ovariektomierten Meerschweinchen gegenuber wiederholten Relaxindosen untersucht (22). Nach den ersten zwei bis drei Gaben steigt die Empfindlichkeit rasch an, und nach einer Periode der relativen Bestandigkeit wird die Empfindlichkeit wiederum vermindert,

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Es wurde verfolgt, wie Östradiol und Relaxin den Einbau markierten Glycins in das Eiweiß des Bindegewebes bei Meerschweinchen-Symphysen und in das Eiweiß anderer Organe beeinflussen (15). Die Unterschiede waren sehr markant. Die Relaxation der Symphysen des Beckens kann durch Anästhesie mit Urethan, Amytal oder Barbital gehemmt werden, ein ähnlicher Effekt wurde durch Durchtrennen des Rückenmarks in der Höhe des zwölften Brustwirbels bei kastrierten weiblichen Meerschweinchen hervorgerufen (25). Die kombinierte Verwendung von Relaxin und Östrogenen zur Relaxation des Beckens wurde auch bei Schafen zur Erkenntnis des Mechanismus dieses Prozesses studiert (8).

Relaxin senkt den Tonus und die spontane Motilität des Uterus. Grundlegende Versuche in dieser Richtung wurden mit wäßrigen Gelbkörperextrakten ausgeführt, und die wirksame Substanz wurde vorläufig als uterusrelaxationauslösender Faktor (*Uterine relaxing factor*, URF) bezeichnet. Später konnte ein ähnlicher Effekt auch bei gereinigten Relaxinpräparaten nachgewiesen werden, und es konnten weitgehend übereinstimmende Bedingungen für die Inaktivierung beider Faktoren ermittelt werden.

Die Erfahrungen mit der Darbietung von Relaxinpräparaten an Menschen sind vorläufig ziemlich beschränkt. Relaxin wirkt im Organismus nicht toxisch, nur an der Einstichstelle tritt Schmerzhaftigkeit auf, und manchmal werden schwächere Entzündungen beobachtet. Das Präparat beeinflußt weder Blutdruck, Puls, Temperatur, Blutbild noch den Corticoidgehalt im Blut. Darbietung von Relaxin allein oder in Kombination mit Cortison beeinflußte bei Patienten mit rheumatischer Arthritis den Krankheitsverlauf nicht (23).

In allerletzter Zeit wurde die Wirkung von Relaxin bei 78 Frauen untersucht, und zwar bei gynäkologischen Patientinnen und bei Freiwilligen; hierbei wurde Relaxin in verschiedenen Dosen i.m. oder i.v. verabreicht, ohne daß Empfindlichkeit gegenüber dem Präparat auftrat (10). Man ermittelte den Einfluß auf die Dilatation des Ureters und die Relaxation der Symphysen des Beckens; beim Studium der Wirkung bei inertem Uterus war keine Interferenz mit Oxytocinpräparaten zu beobachten. Ferner wurde Relaxin bei Patientinnen mit sekundärer Dysmenorrhoe, verbunden mit Endometriose und Adenomyose, angewandt; in diesem Fall verabfolgte man eine einzige intragluteale Injektion von 5000—7500 Meerschweincheneinheiten während der schmerzhaften Phase. Die Zahl der Patienten war jedoch zu gering, als daß definitive Schlußfolgerungen gezogen werden konnten.

Der wäßrige Extrakt der Gelbkörper von Schweinen kommt unter dem Namen *Lutein* oder *Lutuitrin* in den Handel und wird von der Firma Hynson, Westcott und Dunning, Inc., Baltimore, Maryland, hergestellt (3). Ein ähnliches Präparat erzeugt auch Warner-Chilcott Lab., Morris Plains, N. J (4) unter der Bezeichnung *Releasin*.

es erhöht angeblich die Peristaltik des Duodenums, *Duodenin* oder *Inkretin* besitzt angeblich hypoglykämische Wirkung, *Villikinin* aus dem Zwölffingerdarm und den oberen Teilen des Dünndarmes soll die Bewegung der Darmzotten stimulieren, *Peristaltin* die Darmperistaltik, der *Stoff P* wirkt auf die glatte Muskulatur und ist nicht mit Cholin, Acetylcholin oder Histamin identisch.

Secretin. Bayliss und Starling (11) zeigten im Jahre 1902, daß Extrakte aus der Schleimhaut des Dünndarms, die an Hunde intravenös verabreicht wurden, eine starke Vermehrung der Sekretion von Pankreassaft verursachen. Diese Wirkung schrieben die erwähnten Autoren einem im Extrakt anwesenden Wirkstoff zu und nannten ihn *Secretin*. Sie stellten ähnliche Extrakte aus der Darmschleimhaut einer Reihe von Wirbeltieren her und stellten fest, daß in der Secretinaktivität keine Artspezifität existiert. Es wurde angenommen, daß die Schleimhaut das sogenannte *Prosecretin* enthält, welches erst durch Säureeinwirkung in Secretin übergeht, das dann absorbiert wird. Es zeigte sich jedoch bald, daß Säure kein spezifischer Stimulator ist und daß viele Substanzen den gleichen Effekt hervorrufen. Schließlich wurde der Schluß gezogen, daß Secretin in den Zellen der Darmschleimhaut anwesend ist und nur seine Absorption durch eine Reihe verschiedener Stoffe unterstützt wird. Die Stimulierung der Sekretion von Pankreassaft durch Einwirkung von Secretin wurde nicht sofort allgemein angenommen. Pavlov suchte die Sekretion von Pankreassaft auf dem Wege nervöser Reflexe zu erklären, es wurde jedoch erwiesen, daß auch die denervierte Drüse reagiert. Die Verbreitung der Stimulationsanregung auf dem Blutwege wurde an Versuchshunden in der Parabiose nachgewiesen; nach Reizung der Duodenalschleimhaut bei einem Hund wurde bei einem zweiten Hund die pankreatische Sekretion beobachtet.

Eine Reihe von Forschern bemühte sich um die Darstellung von Secretin in reinem Zustand. Der erste Schritt bestand in Versuchen, die Extrakte von Ballaststoffen zu befreien, insbesondere von solchen, die vasodilatorische Wirkung aufweisen. Dale und Laidlaw (16) konnten feststellen, daß der Wirkstoff aus saurer Lösung durch Zusatz von $HgCl_2$ gefällt wird; nach der Zersetzung mittels Schwefelwasserstoff wird die wässerige Lösung nach dem Einengen auf ein kleines Volumen mit Aceton gefällt. Das Produkt liefert ein unlösliches Pikrat, das durch angesäuerten Alkohol zersetzt wird. Stepp (39) erhielt Secretin aus mittels Aceton getrockneten Schleimhäuten durch Extraktion mit 70%igem Alkohol, die Ballaststoffe fällte er durch Erhöhen der Alkoholkonzentration auf 95% und fällte Secretin im Filtrat mittels Äther.

Weaver und Mitarbeiter (40) entfernten die vasodilatorischen Substanzen aus den Schleimhautextrakten nach dem Waschen mit Säure durch Aussalzen des Secretins. Mellanby (35) extrahierte die Schleimhäute mittels Alkohol (abs.), destillierte den Alkohol nach Wasserzusatz ab und adsorbierte das Secretin an Gallensäuren (s. auch

28. Hormone des Verdauungstraktes

Der Verdauungstrakt ist eigentlich kein Bestandteil des innersekretorischen Systems, dennoch wurde festgestellt, daß seine Gewebe Substanzen sekretieren können, welche den Hormonen sehr nahestehen. Es wurden hier gleichfalls keine bestimmten Zellstrukturen gefunden, welche direkt spezifisch für die Sekretion von Hormonen wären Die produzierten Inkrete besitzen, abgesehen von der Schwierigkeit der Unterscheidung ihres Ursprunges, häufig nicht alle Merkmale von Hormonen, trotzdem werden sie für gewöhnlich zu den Hormonen gestellt. Ihre Wirkung ist auf die Lenkung der Tätigkeit und die Koordinierung der Drüsen des Verdauungstraktes (1, 3, 5, 6, 7, 8, 9) neben der parallelen nervösen Regulierung eingestellt. Die Untersuchung des Einflusses der Exstirpation eines bestimmten Gewebes auf Störungen dieser Koordinierung ist durch die zugleich primär verursachten Störungen der Ernährung des Organismus äußerst erschwert, so daß die durch die Störung der Regulierung der Drüsen des Verdauungstraktes auftretenden einzelnen Unzulänglichkeiten völlig verdeckt werden.

Der chemische Charakter der meisten dieser Faktoren ist bisher nicht genügend geklärt. Daher herrschen gewisse Zweifel über die Existenz dieser Faktoren, insbesondere bei den Inkreten, welche nur auf Grund der Wirkungen roher Extrakte vorausgesetzt werden. Als sicher nachgewiesen können bisher nur *Secretin* und *Cholecystokinin* angesehen werden, die wir im weiteren übersichtlich zusammenfassen werden. Die weiteren vorausgesetzten Hormone: *Pancreozymin* (soll die Sekretion der pankreatischen Enzyme regulieren), *Gastrozymin* (soll die Sekretion des Pepsins durch die Magenschleimhaut stimulieren), *Gastrin* (erhöht die Sekretion von Magensaft), *Enterogastron* (hemmt die Magensekretion und die Motilität) sind bisher nicht eindeutig nachgewiesen. *Urogastron* und *Anthelon* sind Extrakte aus Harn, der Ursprung dieser Stoffe ist unbekannt. Urogastron (4) soll eine ähnliche Wirkung wie Enterogastron besitzen, Anthelon verursacht angeblich die Vaskularisierung und Epithelisierung der Magen- und Duodenalschleimhaut; es fehlt angeblich im Harn von Patienten, die an peptischen Geschwüren leiden. Neuestens stellte SVATOŠ (Naturwiss. 45, 523—524, 1958) im Harn einen Faktor fest, der bei den Versuchstieren nach parenteraler Zufuhr die Sekretion der pankreatischen Enzyme erhöht, und nannte ihn *Uropankreozymin*.

Enterocrinin hemmt angeblich die Sekretion der BRUNNERschen Drüsen im Duodenum, *Duocrinin* wurde als das „Hormon" der BRUNNERschen Drüsen bezeichnet;

Secretin ist ein amorphes weißes Pulver, löslich in Wasser, Methanol und Äthanol, unlöslich in Butanol, Aceton und Äther, es ist auch in verdünnten Sauren und Alkalien gut löslich. Secretin geht bei der Dialyse durch die Membran hindurch, es wird verhältnismäßig leicht an verschiedene Adsorbentien adsorbiert, ist jedoch schwer eluierbar. Es ist in Losung insbesondere im alkalischen Gebiet unbestandig, großere Bestandigkeit wurde in saurer Lösung beobachtet. Es handelt sich um einen thermolabilen Stoff, der durch UV-Strahlung inaktiviert wird. Die gleiche Wirkung besitzen Oxydationsmittel. In trockenem Zustand sind Secretinpräparate praktisch bestandig. Es wird empfohlen, die Praparate in einem dunklen und kuhlen Raum zu lagern. ROGERS (38) *gibt an, daß Secretin auch durch Blutserum und Harn inaktiviert wird,* es ist jedoch nicht bekannt, ob es sich um einen enzymatischen Prozeß handelt.

Bei der Elementaranalyse von Praparaten, die aus den kristallinen Pikrolonaten des Secretins dargestellt wurden, wurde folgende Zusammensetzung festgestellt:

	HAMMARSTEN	GREENGARD und IVY
C	46%	52%
H	6%	4,5%
N	12%	20%
S	0,7%	—
O (aus dem Unterschied)	35,3%	23,5%

Bei dem Präparat von HAMMARSTEN wurden durch Reaktion mit salpetriger Säure 7% Aminostickstoff ermittelt. Bei der Analyse von Praparaten nach der Hydrolyse wurde eine Reihe von Aminosäuren festgestellt; diese Angaben findet man neuerdings in der Arbeit von GERSHBEIN und KRUP (20) Daraus kann der Schluß gezogen werden, daß es sich um eine Substanz mit Peptidcharakter handelt, die einige Eiweißreaktionen, z. B. die Biuret-Reaktion, gibt.

Die beiden von HAMMARSTEN und von GREENGARD und IVY dargestellten Pikrolonate sind nicht identisch. Das Praparat von HAMMARSTEN besitzt ein hoheres Molekulargewicht und stellt offenbar eine Verbindung des Secretins mit einem Eiweißstoff dar. *Es gelang tatsachlich, aus diesem Produkt nach der Zersetzung des Pikrolonats* durch Hydrolyse 10 Aminosauren freizusetzen, ohne daß ein Verlust an Aktivitat des Praparates eintritt (10). Das Molekulargewicht wurde mit 5000 angegeben, neuerdings führen GERSHBEIN und KRUP (20) den Wert 12500—14500 an Das kristalline Pikrolonat bildet gelbe Nadeln mit dem Smp. 234—235° C.

Die biologische Bewertung der Aktivitat von Secretinpräparaten wurde an Hunden ausgearbeitet (28) Es wird die Pankreassaftmenge gemessen, die aus einer in den Pankreasausgang eingefuhrten Kanule ausfließt Die *Wirkungseinheit* ist nach IVY diejenige Präparatmenge, die eine Erhohung der Pankreassaftsekretion um 10 Tropfen in der Minute gegenuber der Kontrolle hervorruft. WILANDER und ÅGREN (41) aus der Arbeitsgruppe von HAMMARSTEN verwendeten den Test an urethanisierten Katzen mit geoffnetem Duodenum. Die *Aktivitätseinheit* ist hier die Präparatmenge, die eine Vermehrung des alkalischen Sekrets verursacht, die 0,1 ml 0,1 N Saure bei der

14), welche aus der Losung mittels schwacher Essigsäure gefallt wurden. Den Niederschlag löste er dann in Alkohol und fällte mittels Aceton und Äther. Ivy und Mitarbeiter (29) benutzten die Aussalzmethode, extrahierten dann den Niederschlag mit 90%igem Äthanol und fällten das Secretin nach dem Verdampfen der Lösung mit 5%iger *Trichloressigsaure*.

Ein kristallines Secretinpräparat stellten unabhängig zwei Gruppen von Forschern dar. Hammarsten und Mitarbeiter (23) benutzten die Methode der Fallung des Extraktes mittels Pikrinsäure, die von Pikrat befreite Losung wurde dann elektrodialysiert, wurde dann in das Salicylat ubergeführt und schließlich das kristalline Pikrolonat hergestellt. Greengard und Ivy (21) fraktionierten den mittels Trichloressigsäure aus Schleimhautextrakten erhaltenen Niederschlag durch Lösen in angesäuertem 80%igem Aceton. Durch allmählichen Zusatz von Anilin wurde die Fallung der Ballaststoffe erreicht. Nach deren Abtrennung wurde die Lösung durch Destillation im Vakuum und Verdampfen zur Trockene von Aceton und Anilin befreit. Der Rückstand wurde in Methanol suspendiert, und nach Abtrennen des unlöslichen Ruckstandes wurde mit Äther gefallt. Der erhaltene Niederschlag wurde in Wasser gelost und mittels n-Butanol extrahiert; nach dem Abdestillieren des Butanols wurde mittels Pikrolonsäure gefallt. Das kristalline Pikrolonat wurde umkristallisiert und dann mittels verdunnter Schwefelsäure zersetzt, schließlich wurde mit Äther gefallt. Die freie Base war amorph. Die Reinheit und Wirksamkeit des derart dargestellten Präparates wurde neuerdings im Jahre 1946 (15) bestätigt. Aus dem durch Anilinzusatz gewonnenen Niederschlag kann *Pancreozymin* (22) und aus den Butanolextrakten *Cholecystokinin* als Nebenprodukte der Secretindarstellung bereitet werden.

Friedman und Thomas (18) beschrieben im Jahre 1950 eine neue Methode der Darstellung eines Secretinpraparates, welches frei von vasodilatorischen Stoffen und weder pyrogen noch antigen war. Als Ausgangsmaterial dienten Schweinedärme, und die Extraktion erfolgte mittels 0,13 M HCl ohne Ruhren. Ferner wurde mittels NaCl bis zu einer Konzentration von 32% Salz ausgesalzen Der Niederschlag wurde durch Dekantieren und Filtrieren abgetrennt, dann in Methanol suspendiert und der Extrakt hierauf mittels Aceton gefallt. Der gewonnene Niederschlag wurde mit gesattigter NaCl-Losung ausgelaugt und getrocknet, dann wurde er in angesäuertem [illegible] von 10% [illegible] ziert, mit abs. Aceton und Äther gewaschen und nach dem Trocknen mittels Butanol extrahiert. Der ungeloste Ruckstand wurde mit abs. Aceton und dann mit Äther gewaschen.

Secretinkonzentrate stellten nach modifizierten Verfahren ferner Gershbein und Krup (20) im Jahre 1952 und schließlich Jorpes und Mutt (30) her. Das reinste durch Adsorption an Stearinsaure dargestellte Präparat besaß eine Aktivität von 22000 bis 25000 Katzeneinheiten im mg.

Die Praparate sind in Form eines sterilen Pulvers in Ampullen erhaltlich. Die Losung wird erst vor Gebrauch hergestellt, dies geschieht mit Rucksicht auf die geringe Stabilitat. Von den gebräuchlichen Handelspraparaten fuhren wir hier die folgenden an:

SECRETIN	Eli Lilly USA	(5 mg	= 100 klinische Einheiten)*)
SECRETIN	Sinbio Frankreich . .	(20—30 mg	= 40 klinische Einheiten)
SECRETIN	Mebiol England . . .	(1 Amp.	= 40 klinische Einheiten)
SECRETIN	Spofa ČSR	(20 mg	= 40 klinische Einheiten)

Die Packungen pflegen auch Ampullen mit Losungsmittel zu enthalten.

Cholecystokinin. Okada (36) stellte im Jahre 1914 fest, daß durch Zufuhr von Saure in den Zwolffingerdarm der Tonus der Gallenblase erhoht wird. Boyden (12) zeigte dann, daß Blut von Tieren während der Verdauung eine Substanz enthält, die, wenn sie einem hungernden Tier injiziert wird, Gallenblasenkontraktionen zur Folge hat. Das Blut hungernder Tiere enthält diese Substanz nicht. Ivy und Mitarbeiter (27) wiesen nach, daß es sich um eine Hormonwirkung handelt. Die Entleerung der Gallenblase erfolgt nach Eintritt des Chymus in das Duodenum und das auch dann, wenn Duodenum und Gallenblase nervös isoliert wurden. Die fur diese Wirkung verantwortliche Substanz bezeichneten die Autoren als *Cholecystokinin.*

Lueth und Ivy (34) isolierten dieses Hormon nach der Reinigung des Secretins Der aus dem Trichloressigsäureextrakt aus der Schleimhaut isolierte Niederschlag enthalt sowohl Secretin als auch Cholecystokinin. Nach Auflösen des Niederschlages in 95%igem Alkohol geht das Secretin in Losung, und im Niederschlag verbleibt Cholecystokinin Bei der Reinigung des Secretins nach Greengard und Ivy wurde Cholecystokinin im Butanolextrakt des Filtrates nach dem Anilinniederschlag gefunden Das Hormon wurde bisher nicht in individuellem Zustand isoliert.

Cholecystokinin besitzt ahnliche Eigenschaften wie Secretin, sein isoelektrischer Punkt liegt bei pH 5,0—5,5 zum Unterschied von dem Wert fur Secretin 7,75. Verschieden ist auch die Loslichkeit beider Hormone in Butanol, wie schon angefuhrt wurde. Die hochste Cholecystokininkonzentration wurde im oberen Teil des Dünndarmes festgestellt.

Die biologische Bewertung von Cholecystokininpräparaten erfolgt nach Ivy und Mitarbeiter durch Messung der erhohten Tension der Gallenblase beim Hund nach der Hormoninjektion *Die Einheit der Aktivität* ist jene Menge, die eine Tensionszunahme hervorruft, welche 1 ml Galle in Abwesenheit von vasodilatorischen Stoffen entspricht. Der Test wurde auch an Gallenblasen in vitro, an Meerschweinchengallenblasen in situ und in vitro und sogar auch an Gallenblasen decapitierter Frosche untersucht (s. 9)

*) 1 klinische Einheit = 1 Ivy-Einheit.

Neutralisierung auf Methylorange äquivalent ist. Eine IVY-Einheit entspricht 20 HAMMARSTEN-Einheiten. BURN und Mitarbeiter (13) *standardisieren Secretin*präparate mit Hilfe der Methode der Pankreasfistel bei Katzen. DOUBILET (15) modifizierte die Methode wiederum an Hunden einerseits durch Unterbinden der Gallengänge, andererseits durch Anwendung eines Tropfenzählers, eine andere Modifikation geben GERSHBEIN und Mitarbeiter an (19).

Nach anderen Verfahren werden als Versuchstiere Kaninchen oder auch Ratten verwendet (s. 9, 26); es wurde auch der Versuch unternommen, Secretinpräparate papierchromatographisch zu bewerten (32).

Secretin ist vorwiegend in der Schleimhaut des Duodenums enthalten, seine Lokalisierung ist hier jedoch nicht beschränkt. Im Jejunum nimmt seine Konzentration allmählich ab; *es wird angegeben, daß dieser Faktor von den argentophilen* Zellen des Dünndarms abgeschieden wird. Die wesentlichste physiologische Funktion des Secretins besteht in der Stimulierung der Zellen des Pankreasgewebes zu erhöhter Sekretion von Verdauungssaft (2). Es wird die Sekretmenge vermehrt und keineswegs die Enzymmenge, für diese Funktion wird ein weiterer Faktor, das *Pancreozym* vorausgesetzt (25). Die Beziehung zu der Menge abgeschiedenen Bicarbonats ist nicht völlig klar, obwohl auf dieser Wirkung eine der vorgeschlagenen Titrationsmethoden für Secretin beruht. Die sogenannten extrapancreatischen Wirkungen des Secretins sind leicht von seinen Begleitstoffen in nicht völlig reinen Präparaten abzuleiten.

Nach der Feststellung, daß weder Atropin noch Ergotamin die Secretinwirkung hemmen, wurde angenommen, daß die Wirkung dieses Faktors auf das Gewebe unmittelbar, ohne Mitwirkung des autonomen Nervensystems *erfolgt.* OLEANDROV (37) führt jedoch an, daß Secretin eine gangliotrope Wirkung besitzt. Mit der pharmakodynamischen Wirkung des gereinigten Secretins *befassen sich neuerdings* die Arbeiten von LENZI und LABÓ (33). Secretin besitzt keine Wirkung auf die Sekretion des Magens und auf den Blutzuckerspiegel

Pancreozymin begleitet im *allgemeinen* Secretin in nicht genügend gereinigten *Präparaten und verursacht die Sekretion von Amylasen, Lipasen* und von Trypsin [illegible] ...phatase im Pankreas- [illegible]

traten. Durch die Anwesenheit dieses Faktors können einige Widersprüche in den Angaben verschiedener Autoren über die Eigenschaften des Secretins erklärt werden.

Secretin kann in der klinischen Praxis als diagnostisches Mittel für die Untersuchung des funktionellen *Zustandes des Pankreas verwendet werden.* Das Präparat wird intravenös verabreicht, und die Auswertung erfolgt durch Entnahme einer Saft- [illegible]

wirkungen hervorrufenden Stoffen sind.

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Cholecystokinin verursacht einerseits die Kontraktion der glatten Muskulatur der Gallenblase, andererseits eine Schwächung des Schließmuskels. Die Relaxation des *Schließmuskels nach der Hormonverabreichung* wird durch *Adrenalin, Atropin*, Acetylcholin oder Prostigmin nicht beeinflußt. Das Präparat könnte in der Diagnostik für die Bestimmung der Funktion der Gallenblase (31) in Verbindung mit dem Test von Graham und Cole auf die Entleerungsfähigkeit verwendet werden. Vorderhand stehen jedoch keine geeigneten Präparate für den täglichen Gebrauch zur Verfügung.

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VI. Hormonwirkung

Nachdem wir die einzelnen Hormongruppen besprochen und in Kürze ihre Eigenschaften kennengelernt haben, seien nun einige allgemeinere Beziehungen dargelegt, die in den früheren Abschnitten nicht behandelt werden konnten, damit der Zusammenhang nicht unterbrochen und der Erklärung nicht vorgegriffen wurde. Bei einigen Hormonen wurden die Anschauungen über ihren Wirkungsmechanismus bereits kurz erwähnt. Das Gesamtbild in dieser Frage ist bisher noch nicht ganz klar, dennoch waren bereits einige Anzeichen zu finden, die vor allem zum Verständnis der biochemischen durch Hormone vermittelten Impulsübertragung im Steuerungssystem des Organismus dienen können.

Aus der allgemeinen Biochemie ist bekannt, daß die im Organismus stattfindenden Stoffwechselreaktionen von Enzymen katalysiert werden. Diese Biokatalysatoren stellen allerdings kein stabiles, im voraus feststehendes System dar, sondern unterliegen auch dem Stoffwechsel; sie entstehen durch Adaptation. Ihre Aktivität wird von zahlreichen Faktoren gesteuert, und sie werden schließlich wieder durch Abbau inaktiviert. Der Stoffwechsel verschiedener Gewebe trägt typisch zyklischen Charakter, wobei eine Reihe simultaner Kettenreaktionen zur Geltung kommt. Diese finden schließlich auch in den physiologischen und morphologischen Veränderungen in Geweben und Organen ihren Ausdruck, die wir summarisch vielfach als Folge der Wirkung eines bestimmten Hormons bezeichnen.

Daher wurde der Einfluß der Hormone auf die Gewebsenzyme in letzter Zeit sehr intensiv untersucht, und im folgenden seien die dabei erzielten Ergebnisse dargestellt. Die spezifische Wirkung mancher Hormone auf bestimmte Gewebe und Organe beruht offenbar auf der Beeinflussung der Enzymaktivität der vorhandenen Enzymsysteme. Damit hängt auch die Wirkung der Hormone auf das Wachstum der Gewebe und schließlich die hochinteressante Frage des Einflusses der Hormone auf die bösartige Wucherung der Gewebe eng zusammen. Mit dem Abschnitt über den Einfluß der Hormone in der Carcinogenese und über die Wachstumshemmung von Tumoren in Organen, die ausgesprochen unter dem Einfluß der Hormonsteuerung stehen, schließt diese Abhandlung. Auch auf diesem Gebiet besteht bisher keine völlige Klarheit, wie es bei unserer unvollständigen Kenntnis der zum Entgleisen des koordinierten Gewebswachstums führenden Prozesse vorläufig gar nicht anders sein kann.

Die Hemmwirkung der Hormone auf die bösartigen Geschwulste von Prostata und Brustdrüse wurde bereits in dem Abschnitt über Östrogene bzw. Androgene erwähnt. Durch geeignetes Antagonisieren der eigenen Produktion an Hormonen, welche die Tätigkeit dieser Organe beeinflussen, konnten gewisse positive, leider aber nicht anhaltende Ergebnisse erzielt werden.

29. Hormone und Enzymsysteme

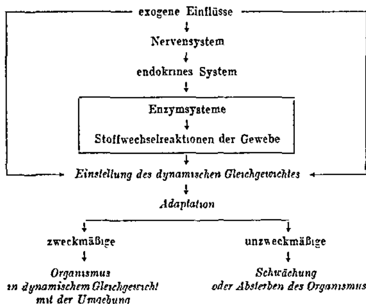
Enzyme, Vitamine und Hormone werden bekanntlich als *Biokatalysatoren* der Stoffwechselvorgänge im Organismus bezeichnet. Während die Beziehung der Vitamine zu den Fermenten heute bereits grundsätzlich geklärt ist und zahlreiche Belege dafür gefunden wurden, daß die Vitamine Muttersubstanzen für Coenzyme sein können, gibt es bei den Hormonen keine derartige einfache Beziehung.

Dennoch bestehen keine Zweifel darüber, daß die Hormone ihren regulierenden Einfluß im Organismus durch Interaktion mit den Enzymsystemen bzw. Modifikationen der Aktivität der einzelnen Enzyme ausüben. EULER und ZONDEK zeigten bereits im Jahre 1934, daß die Inaktivierungskurve der Gonadotropine ebenso verläuft wie die Kurven der typischen Enzyme. Daraus darf allerdings nicht geschlossen werden, daß die Hormone Stoffwechselreaktionen durch direkte Einwirkung katalysieren würden, d. h. durch vorübergehende Bindung mit den einzelnen Stoffwechselprodukten, wie dies bei den Enzymen der Fall ist Es hat jedoch den Anschein, daß die Hormone zumeist auf den Verlauf einer einzigen „Schlüssel"-Reaktion im System einwirken, allerdings sekundär durch Beeinflussung der entsprechenden enzymatischen Reaktion.

Das Schema der Eingliederung der Hormone in das gesamte Regulierungssystem des Organismus können wir folgendermaßen veranschaulichen (s. S. 599).

Es ist bis heute nicht genau bekannt, ob die Hormone allein oder erst in Verbindung mit anderen Substanzen wirken. Von Thyroxin ist z. B. bekannt, daß es im Organismus auch an Eiweiß gebunden vorkommt, ob nun als Thyreoglobulin in der Schilddrüse, was offenbar seine Depotform darstellt, oder an das Bluteiweiß gebunden. Es wurde festgestellt, daß Gewebshomogenate von Rattenhirn in vitro einen größeren Sauerstoffverbrauch nach Zusatz von Thyreoglobulin aufweisen, während Thyroxin unter ähnlichen Bedingungen keine Steigerung bewirkt (64) Die Ansätze wurden in Gegenwart von Glucose, Brenztrauben- oder Bernsteinsäure bebrütet. Eine ähnliche Steigerung des Sauerstoffverbrauchs beobachtete man bei Homogenaten von Ratten, denen Thyroxin und Aneurin verabfolgt wurden, gegenüber Kontrollpräparaten aus dem Gehirn von Ratten, die nur Aneurin erhielten

In den Geweben oder Organen treten nach der Einwirkung von Hormonen Veränderungen in den Aktivitäten verschiedener Enzyme auf. Man verfolgte z B die Enzymaktivitäten in den Gelbkörpern während der Gravidität und Lactation bei Ratten, ferner nach der Kastration im Blut. Durch Verabfolgung der entsprechenden



Hormone der entnommenen Druse konnte der Spiegel der Enzymaktivitaten oft auf die Norm gebracht werden. Die Hormonwirkung kann sowohl in der Anregung der Enzymwirksamkeit als auch in der Hemmung des Systems oder schließlich in der Unterbindung der Hemmung des Enzymsystems durch einen anderen Faktor beruhen. Derartige empirische Befunde wurden bereits in einer Reihe wertvoller zusammenfassender Arbeiten veröffentlicht (s 1—14). Indes kann aus den fragmentarischen Kenntnissen über den Einfluß der einzelnen Hormone auf die Enzymreaktionen nur sehr schwer auf den gesamten Wirkungsmechanismus eines bestimmten Hormons geschlossen werden.

Biogenese und Inaktivierung der Hormone in den Geweben sind allerdings anderseits auch das Ergebnis enzymatisch katalysierter Reaktionen. Die Hormone konnen also auch das „Substrat" fur verschiedene Enzyme darstellen Einige dieser Beziehungen wurden bereits beim Stoffwechsel der Steroidhormone erwahnt (s. S. 395). Das Steroidsubstrat kann auch bei der adaptiven Bildung verschiedener Enzyme induzierend wirken. Die Wechselbeziehungen der Hormone und Enzyme können entweder durch Erfassung der empirischen Befunde uber den Einfluß der Hormone auf die einzelnen Stoffwechselprozesse, z. B. Mitwirkung beim Kohlenhydratstoffwechsel u. ä. m , oder durch Zusammenfassung der festgestellten Wirkungen auf die Enzyme bei den einzelnen Hormongruppen dargestellt werden. Der Kurze halber wird im folgenden von dieser zweiten Möglichkeit Gebrauch gemacht

Die Wirkung der Hormone der *Schilddruse* auf die Aktivitäten verschiedener Enzyme in den Geweben wird schon Jahre hindurch sehr eingehend untersucht. Es

wurden insbesondere *die einzelnen metabolischen Wirkungen des Thyroxins in vitro* untersucht (39, 48) und festgestellt, daß das Hormon vor allem verschiedene Redoxsysteme in den Geweben beeinflußt. Das Hormon erhöht die Sauerstoffaufnahme durch das Gewebe verschiedener Organe, namentlich wenn es den Tieren in vivo verabfolgt wurde; bei der Applikation direkt in vitro waren die Ergebnisse uneinheitlich und hingen oft wesentlich von der angewandten Hormongabe ab. Niedrigere Hormonkonzentrationen erhöhen die Geschwindigkeit der aeroben Phosphorylierung, größere Dosen wirken jedoch im entgegengesetzten Sinne (54). Nach Thyreoidektomie beobachtete man *verminderten Gehalt an Cytochrom C in den Muskeln bei Ratten*, ähnlich nach Zufuhr des antithyreoid wirksamen Methylthiouracils (74); Thyroxin erhöhte jedoch den Cytochromgehalt. Im Lebergewebe von mit Zusatz von getrockneter Schilddrüse gefütterten Ratten wurde höhere Aktivität der Succinoxydase und Cytochromoxydase festgestellt (73). Das Hormon ist offenbar durch Interaktion mit den SH-Gruppen des Enzymkomplexes wirksam (47).

Dagegen wurde in den Geweben des Zentralnervensystems nach *Thyroxineinwirkung in vitro* Abnahme des Sauerstoffverbrauchs beobachtet (55). *Thyroxin hemmt die Atmung von Stierspermatozoen und regt die Glykolyse schwach an* (s. 48). Bei Rattennierenhomogenaten wurde starke Hemmung der Oxydation von Glutamat nach der Einwirkung des Hormons in der Dosis $1{,}3 \cdot 10^{-6}$ M festgestellt; das Hormon hemmt ferner die Oxydation der β-Oxybuttersäure und einiger Fettsäuren bei niedriger Konzentration der Enzyme. Starken Einfluß übt es auch auf den Gehalt an Coenzym A in verschiedenen Geweben *in vivo* aus, während *in vitro* diese Veränderungen nicht zu beobachten waren (70, 63). Thyroxin beeinflußt angeblich auch die Umwandlung von Carotin in Vitamin A (28); demnach würde die Schilddrüse die Verwertung dieses Provitamins im Organismus bedingen. Bei experimentellem Hyperthyreoidismus beobachtete man erhöhte Hexokinaseaktivität in den Muskeln (67), was mit dem erhöhten Kohlenhydratstoffwechsel zusammenhängt.

Mit diesem Enzym ist auch die Wirkung des Insulins verknüpft Bei Ratten mit Alloxandiabetes wurde in den Muskeln verminderte Hexokinaseaktivität festgestellt, die durch Insulin wieder hergestellt wird (20, 21, 38, 61). Insulin hebt auch die Hemmung des Enzyms auf, die durch das somatotrope Hormon des Hypophysenvorderlappens allein oder in Verbindung mit Corticoiden ausgelöst wurde. Weitere Versuche über den Einfluß von Insulin auf die Verwertung der Glukose wurden mit Lebergewebe und auch mit Präparaten des Zwerchfells von Versuchstieren durchgeführt Auch verfolgte man den Effekt von Adrenalin, Glucagon und Somatotropin auf diesen Vorgang Die Hexokinase katalysiert die Überführung des Phosphorsäurerestes von ATP an Glucose, wobei Glucose-6-phosphat gebildet wird; diese Schlüsselreaktion zur Verwertung von Zucker im Organismus wird von zahlreichen Faktoren beeinflußt; wie angedeutet, *ist hier auch das Insulin beteiligt. Selbst scheint* es jedoch die Phosphorylierung des Zuckers nicht unmittelbar zu beeinflussen.

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herabgesetzt, Zufuhr von Hypophysensomatotropin erhoht hingegen die Bildung. In Verbindung mit Cortison bewirkt es erhohte Ketogenese auch nach mehr als 50 Tagen nach der Operation des Versuchstiers. Durch Adrenalin aktivierte Glykogenolyse wurde sowohl im Muskelgewebe (22, 36, 49) als auch bei Leberschnitten in vitro ermittelt (17). Unter bestimmten Bedingungen stellte man jedoch nach Adrenalin bei hungernden Ratten auch Glykogenablagerung in der Leber fest (46)

Adrenalin steigert die Geschwindigkeit der intrazellularen Oxydationsvorgange, wie in vitro an Gehirn- und Muskelgewebe bei verschiedenen Tierarten festgestellt wurde (40, 53). Adrenalin wird durch das Indophenol-Cytochromsystem leicht reduziert, das entstehende o-Chinon kann als Wasserstoffubertrager dienen. Das System ist z. B. an der Dehydrierung der Bernsteinsäure zu Fumarsaure und ihrer Umwandlung in Äpfelsaure beteiligt Die das Adrenalin oxydierende Aminoxydase wurde in verschiedenen tierischen Organen, u. a. auch im Nebennierenmark gefunden. Einspritzen von Adrenalin bei Versuchstieren bewirkt merkliche Herabsetzung der Indophenoloxydaseaktivität im Nervensystem (58).

Nach Insulinzufuhr beobachtete man bei Hunden vorubergehend Erhohung des Thiaminpyrophosphatspiegels (Coenzym I, Cocarboxylase) im Blut, die von Senkung des Spiegels der anorganischen Phosphate begleitet ist (32). Ähnlich ermittelte man bei Ratten gesteigerte Biosynthese der Cocarboxylase in der Leber (66, 75) bei gleichzeitiger Zunahme der Konzentration von ATP, das als Donator der Phosphorsaurereste bei der Coenzymsynthese dient

Wahrend Insulin die Ketogenese unterdruckt, erhoht das *Somatropin* der Hypophyse dagegen die Menge der Ketontrager in der Leber, wie die Bestimmung an normalen und hypophysektomierten Tieren nach Hormonzufuhr ergab Mit Hilfe des Somatotropinpraparates Armour gelang es, auch in vitro nach Zusatz des Hormons zum Inkubationsmedium bei uberlebenden Leberschnitten die Ketogenese zu erhöhen (s 71). Die Hemmung der Hexokinase durch Einwirkung von Hypophysensomatotropin wurde bereits beim Insulin erwahnt CORI (2) zieht bei der hormonalen Steuerung des Blutzuckerspiegels zumindest zwei daran beteiligte Enzymsysteme in Erwägung; das eine katalysiert die Bildung von Zucker aus Glykogen, das ist die Leberphosphorylase, und das zweite, die Muskelhexokinase, sorgt fur seine Utilisation

Somatotropin hangt ferner auch mit den Oxydationsfermenten der Leber (16) und Muskeln (65) zusammen Es wurde der Gehalt an D-Aminosaurenoxydase in der Leber und an Succinodehydrogenase im Muskelgewebe bei Ratten unter dem Einfluß von Somatotropin verfolgt. Hypophysektomie fuhrt Abnahme der Enzymaktivitat herbei, dies hängt aber auch mit der verminderten Schilddrusenfunktion zusammen Die Biosynthese beider Enzyme verlauft mit dem Wachstum des entsprechenden

Gewebes parallel (33). Somatotropin bewirkt ferner Steigerung der Aktivität der alkalischen Phosphatase im Plasma hypophysektomierter Ratten, ACTH hingegen setzt die Aktivität des Serums herab, und zwar bei normalen sowie bei hypophysektomierten Ratten (51).

Hinsichtlich der Wirkung der *Corticoidhormone* auf die Aktivität der Phosphatasen wurde bei Ratten festgestellt, daß Corticosteron oder Gesamtnebennierenextrakt die Aktivität herabsetzt, Cortexon hingegen erhöht (80). KOCHAKIAN fand, daß Corticoide mit der Sauerstoffunktion in Stellung 11 die Aktivität der alkalischen Phosphatase der Leber erhöhen, daß diese Wirkung jedoch nicht mit der glucotropen Aktivität der Steroide in Einklang steht. Einige Androgene setzen den Gehalt an der alkalischen Phosphatase in den Geweben herab und erhöhen umgekehrt die Aktivität der sauren Phosphatase in den Nieren sowohl bei normalen als auch bei kastrierten Mausen (45). Alle Steroide, die Nierenvergrößerung bewirken, setzten die alkalische Phosphatase herab und erhöhten die Aktivität der sauren Phosphatasen. Bei der Untersuchung des Einflusses der Androgene auf die Arginase der Nieren beobachtete man bei niedrigeren Hormonkonzentrationen Abnahme der Enzymaktivität, in größeren Konzentrationen hingegen Steigerung, und zwar im Verhältnis zu der hervorgerufenen Nierenvergrößerung. Insbesondere Steroide mit der Methylgruppe in Stellung 17 waren in dieser Hinsicht stark wirksam. Einen bedeutenden Einfluß üben jedoch Alter und Geschlecht der Versuchstiere aus (68). Hingegen ändert sich der Arginasespiegel der Leber weder durch Kastrieren noch durch Einwirkung der Steroidhormone; wenig ausgeprägte Ergebnisse wurden auch bei der Arbeit mit Intestinalgewebe gewonnen.

Mit Untersuchungen der Beziehung zwischen der renotropen und androgenen Aktivität der Steroide beschäftigte sich vor allem KOCHAKIAN (8); gleichzeitig untersuchte er auch den Einfluß der Androgene und verwandten Steroide auf die Aktivität der Gewebsenzyme, namentlich derjenigen, die am Proteinstoffwechsel beteiligt sind. Der anabolische Effekt dieser Steroide wurde im Abschnitt über die Androgene erwähnt, sein Mechanismus ist jedoch bis jetzt ungeklärt.

Sehr eingehend wurde der Einfluß der Steroidhormone auf die Oxydoreduktionsvorgänge in den Geweben untersucht. 3-Ketosteroide können starke Hemmung z. B der aeroben und anaeroben Oxydation des α-Glycerinphosphats bei Hefen verursachen, während 17- oder 20-Ketosteroide in dieser Richtung bereits schwächer wirksam sind. Die Anwesenheit der Seitenkette an C_{17} des Steroidmoleküls schwächt die durch die vorhandene 3-Ketogruppe in demselben Molekül des Steroidhormons hervorgerufene Hemmwirkung ab. Die Hemmung der Oxydation von Glycerinphosphat in tierischen Geweben durch Einwirkung von Steroidhormonen wird offenbar durch die vorzugsweise Interaktiom verschiedener Gewebskomponenten mit den Steroiden gedämpft (37). Das synthetische Östrogen *Diäthylstilböstrol* wirkt als Wasserstoffübertrager im Dehydrogenasesystem, wie in Gegenwart von MnO_2 als

letztem Wasserstoffacceptor ermittelt wurde. Bei den Hefedehydrogenasen wirkt es kompetitiv wie das System *Hydrochinon-Chinon* gegenuber Methylenblau oder Ferricyanid. In allerletzter Zeit wies TALALAY, P. mit Mitarbeitern (J. biol. Chem. 233, 886, 1958) nach, daß die 3α-Oxysteroide als *Coenzyme* der Wasserstoffubertragung bei Diphospho- und Triphosphopyridinnucleotiden wirken konnen.

Nach Injektion von 2,5 μg Östron wurde die Geschwindigkeit der anaeroben Glykolyse des Rattenuterus wahrend des Proostrus und bei kastrierten Ratten verdoppelt. Nach Zusatz des Hormons in vitro war jedoch kein Einfluß auf die Milchsäurebildung festzustellen. In den Gelbkorpern gravider oder pseudogravider Ratten wurde hohe Aktivität der Succinodehydrogenase (57) festgestellt, deren Biosynthese mit dem luteotropen Effekt des Hypophysenvorderlappens in engem Zusammenhang steht. Die Phenolgruppen der Östrogene hemmen die Enzymaktivitat, wahrend die Hydroxylgruppen der Androgene offenbar ohne Einfluß sind (11). Mittels Diathylstilböstrol bewirkte man Hemmung des Sauerstoffverbrauches in den Mitochondrien verschiedener Tumoren; Testosteron oder Progesteron verhinderten die Hemmung (79). Es wurde auch der Einfluß der Östrogene auf die Dehydrogenasen des Lebergewebes untersucht. Die ostrogene Wirksamkeit verschiedener synthetischer Stilbenderivate ist nicht parallel zu ihrer Hemmwirkung gegenüber Succinodehydrogenase (18) Durch Acetylierung der Phenolgruppen nimmt die Hemmaktivität der Derivate gegenuber den Enzymen stark ab (29).

Die Äpfelsäuredehydrogenase und die Succinodehydrogenase der Leber werden in Gewebshomogenaten auch durch androgene Hormone gehemmt, wie durch Messung nach der Warburg-Technik festgestellt wurde; es tritt hier irreversible Verknüpfung des Enzyms mit dem Inhibitor ein (41). Zu den Versuchen wurden wasserlösliche Derivate der Androgene verwendet. Testosterondiäthylaminoäthylcarbonat bildet mit dem Enzym einen Komplex, wobei 2 Mol. des Inhibitors mit einem Enzymmolekül zusammentreten (50).

Hemmwirkung der Steroidhormone wurde in Leberhomogenaten auch gegenüber Cholinoxydase festgestellt. Die veresterten Hormone besaßen bedeutend schwächere Hemmwirkung. Am wirksamsten waren in dieser Hinsicht Testosteron, Progesteron und Cortexon, während Östradiol und Cortison das Enzym nur schwach hemmten (27). Ferner wurden die Veränderungen in der Aktivität der Succinodehydrogenase und Cytochromoxydase nach Einwirkung von Androgenen und Östrogenen im Prostatagewebe und in Samenblasen bei Ratten untersucht (23). Nach Kastrierung wurde Abnahme der Enzymaktivität festgestellt, die Zufuhr von Androgen stellt den Spiegel wieder her. Nach Kastration wurden im Ventrallappen der Prostata bei Ratten Abnahme der Äpfelsäurehydrogenase, Aconitase und Fumarase, dagegen erhöhte Aktivität der Milchsäuredehydrogenase festgestellt. Der Spiegel von Isocitronensäuredehydrogenase, Glucose-6-phosphatase und Enolase wurde nicht beeinflußt. Testosteron bringt den veränderten Spiegel wiederum auf die Norm zurück (81).

Die natürlichen Östrogene zum Unterschied von den synthetischen Derivaten und den übrigen Geschlechtshormonen regen die Aktivität der Histaminase an. Bei diesem Wirkungsmechanismus kommt offenbar dem Flavinadenin-dinucleotid eine wichtige Aufgabe zu. Diese Frage könnte beim Studium der Vorgänge bei der normalen und toxämischen Schwangerschaft von Bedeutung sein; bereits im 8. Schwangerschaftsmonat kann man einen auffallenden Anstieg der Histaminaseaktivität im Blutserum beobachten (42).

Ferner wurde die Beziehung der Steroidhormone zur Aktivität der β-Glucuronidase in verschiedenen Geweben untersucht. Die Östrogene erhöhen den Enzymspiegel in den Geweben der sekundären Geschlechtsorgane, beeinflussen jedoch nicht die Aktivität des Enzyms in Leber, Nieren und Milz. Progesteron bewirkt in beiden Fällen Abnahme des Spiegels der Enzymaktivität. Es besteht eine gewisse Beziehung zwischen dem Enzymgehalt im Serum und der Ketosteroidmenge im Harn, die in der konjugierten Form der Glucuronide ausgeschieden wird (19). Kastration bei geschlechtsreifen männlichen Ratten war ohne Einfluß auf die Aktivität der Glucuronidase in Prostata und Samenblasen, bei Weibchen tritt jedoch nach Kastration eine starke Abnahme der Enzymaktivität in den Geweben ein, die durch Östrogenzufuhr aufgehoben werden kann (44). Auch bei Männchen bewirkte Östrogenzufuhr angeblich Steigerung der Enzymaktivität in beiden erwähnten Organen. Testosteron rief bei kastrierten Weibchen Erhöhung des Glucuronidasespiegels im Uterusgewebe hervor. Progesteron dämpfte die stimulierende Wirkung der Östrogene auf die Aktivität des Ferments. β-Glucuronidase spielt im Organismus der Säugetiere eine wichtige Rolle. Das Enzym katalysiert die primäre Reaktion im Mechanismus der Östrogeneinwirkung auf die sekundären Geschlechtsorgane (31). Mittels Testosteronpropionat konnte angeblich Zunahme des Enzyms in den Nieren von Mäusen beiderlei Geschlechts ausgelöst werden; Stilböstrol hob diese Stimulierung auf (30). Nach Zufuhr von Stilböstrol beobachtete man deutlichen Anstieg der Glucuronidaseaktivität in der Hypophyse bei Ratten (56).

Bei männlichen Ratten, denen Testosteronpropionat verabreicht wurde, ermittelte man in den Testes einen deutlich höheren Hyaluronidasespiegel als bei der Kontrollgruppe (62). Das Enzym wird von Cortison und auch von 11-Dehydrocorticosteron gehemmt, Hemmung durch Corticoide wurde auch bei Versuchen in vitro beobachtet (60).

Verzár (13, 76, 77) beschäftigte sich mit der Untersuchung der Beziehungen zwischen den Corticoiden und der Phosphorylierungsfähigkeit der Gewebe gegenüber Glykogen. Das Muskelgewebe adrenalektomierter Tiere besitzt geringere Phosphorylierungsaktivität, die jedoch durch Corticoide angeregt werden kann. Montigel (59) führt an, daß Cortexon eine wichtige Komponente des die Phosphorylierung von Glykogen im Muskelgewebe bewerkstelligenden Enzymsystems ist. Diese Ansicht fand jedoch keine Bestätigung. Cortison setzt die Aktivität der Phosphorylase bei

Kaninchen herab, hingegen regt Adrenalin die Aktivitat des Enzyms an (43). Bei der Untersuchung des Cortisoneinflusses auf den Kohlenhydratstoffwechsel in der Leber wurde Abnahme an Hexosephosphat ermittelt (24); bei der Arbeit mit Leberhomogenaten bei Ratten bestimmte man die Aktivität der Glucose-6-phosphatase nach vorangehender i m. Injektion des Hormons an Versuchstiere. Nach Verabfolgung von Cortison tritt Erhöhung des Blutzuckers und der Phosphataseaktivität in der Leber ein (78).

Nach Adrenalektomie fand man bei Ratten verminderte Aktivitat der Cytochromoxydase in verschiedenen Geweben und Abnahme der Konzentration von Cytochrom C in Nieren und Leber. Nebennierenrindenextrakt vermag diese Veränderungen auszugleichen (72) HAYANO (34, 35) verfolgte die Hemmung der D-Aminosäureoxydase unter dem Einfluß von Corticoiden, insbesondere Cortexon. Das Steroid wirkt offenbar durch Interaktion mit der Apoenzymkomponente des Flavoproteins. Cortexon hemmte ferner Tyrosinase, Urease, Ascorbinsaureoxydase, Lipase und Transaminase; fördernde Wirkung wurde hingegen bei Glutaminase, Trypsin und Hefecarboxylase gefunden. DIRSCHERL mit Mitarbeitern (25) untersuchte ferner den Einfluß der Steroidhormone auf die Hefecarboxylase und auch auf das Enzym des Herzmuskels bei verschiedenen Steroidkonzentrationen sowie die Beeinflussung der D-Aminosäureoxydase durch verschiedene Steroidkonzentrationen (26).

Auf Grund histochemischer Untersuchungen wurden in letzter Zeit die Aktivitäten verschiedener Corticoide mit *Aldosteron* gegenuber verschiedenen Gewebsenzymen verglichen Man stellte Stimulierung der sauren Phosphatase in den Nierentubuli und im Dunndarm fest, Inhibition der alkalischen Phosphatase der Nieren, nicht jedoch in Dünndarm und Testes, ferner Stimulierung der Lipaseaktivität u. ä. m. (52) Im Vergleich mit den ubrigen Corticoiden unterscheidet sich Aldosteron auch in dieser Hinsicht ziemlich stark; in der Beeinflussung der Enzymaktivitaten ahnelt ihm Cortexon am meisten. Nach Verabfolgung von Aldosteron an Ratten wurden verschiedene Organe auf die Anwesenheit von Succinodehydrogenase histochemisch untersucht Das Enzym konnte dann auch in Geweben aufgefunden werden, in denen es normalerweise nicht vorkommt (15).

Wie aus den angefuhrten Beispielen der Hormonwirkung auf Enzymsysteme der Gewebe des tierischen Organismus hervorgeht, können aus diesen ermittelten Beziehungen vorlaufig keine allgemeineren Schlußfolgerungen gezogen werden, da das Gesamtbild zu fragmentarisch ist. Dieses Gebiet stellt jedoch eines der interessantesten Kapitel der Biochemie dar

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79. [illegible]
80. [illegible]
81. [illegible]

30. Hormone und bösartige Geschwülste

Das Wachstum der einzelnen Gewebe des Organismus verläuft normalerweise harmonisch in Übereinstimmung mit den Geweben der Umgebung, es ist einem regulierenden System und dem Bedarf des Organismus untergeordnet. Vom Wachstum in der Entwicklungsphase des Organismus abgesehen, ist dieser komplizierte und bisher völlig unaufgeklärte Vorgang nach erreichter Reife vornehmlich auf die Regeneration der Gewebe und auf die funktionelle Anpassung an die veränderten Bedingungen und Ansprüche hinsichtlich der Leistung des Organismus beschränkt. Alternde und absterbende Zellen werden durch neue ersetzt, das geschädigte Gewebe wird zu einem gewissen Grad zur vollen funktionellen Leistungsfähigkeit ergänzt. Dieses Wachstum kann als *Regenerationswachstum* bezeichnet werden. Hinsichtlich der Funktion der Anpassung finden sich Beispiele bei den hypertrophischen Adaptionen, was die Reaktion auf die erhöhten Ansprüche an die Tätigkeit eines bestimmten Gewebes bedeutet, ferner bei Wachstumsprozessen in verschiedenen Geweben während des Sexualzyklus und während der Gravidität bei Berücksichtigung des Organismus der Mutter.

Die bösartige Gewebswucherung entzieht sich den erwähnten Wachstumsreaktionen des Organismus; es ist eine für den ganzen Organismus unzweckmäßige Reaktion auf zumeist bisher unbekannte Reize. Es konnte zwar festgestellt werden, daß eine Reihe verschiedener chemischer Substanzen *cancerogen* wirkt, so z. B. verschiedene höhere aromatische Kohlenwasserstoffe, Azofarbstoffe usw. Ferner können wiederholte Reizung der Gewebe durch stärkere Dosen durchdringender Strahlung und andere wiederholt einwirkende Einflüsse auf die Gewebe unter gewissen Umständen maligne Wucherung hervorrufen. Es bleibt jedoch unentschieden, ob weitere bisher unbekannte, für die Entstehung bösartiger Geschwülste entscheidende Einflüsse bestehen und was am wichtigsten ist, ob die Empfänglichkeit für diese Reize durch das gestörte regulierende System des Organismus gegeben ist. Der Großteil des gesammelten empirischen Materials über das Problem der bösartigen Geschwülste betrifft eher die morphologischen, histologischen und metabolischen Folgeerscheinungen als die Umstände, die für das Eintreten dieser Entgleisung des Gewebes aus der Unterordnung des regulierenden Systems des Organismus entscheidend sind.

Obwohl bösartige Geschwülste vom frühen Kindesalter an bei jeder Altersstufe auftreten, sind sie doch viel häufiger im Alter, da endokrine Störungen infolge Er-

schöpfung verschiedener Organe auftreten. Das endokrine System hätte also von vornherein eine gewisse Beziehung zu den bösartigen Geschwülsten; die Hormone als Überträger der Steuerungsimpulse hängen dann vor allem mit den malignen Geschwülsten derjenigen Organe zusammen, auf die sie normalerweise vorherrschend einwirken. So werden z. B. bei der Krebserkrankung eines der sekundären weiblichen Geschlechtsorgane die gonadotropen und östrogenen Hormone, welche entweder direkt oder indirekt das „Wachstum" und die Entwicklung dieser Organe bewirken, den pathologischen Zustand verschlimmern (51), ähnlich die Androgene bei den bösartigen Geschwülsten der sekundären männlichen Geschlechtsorgane. Faktoren, die das „Wachstum" dieses Organs hemmen, werden hingegen günstige Wirkung aufweisen.

Viel Aufmerksamkeit wurde der Frage der *cancerogenen* Wirkung der Hormone (6, 12, 17, 20), namentlich der Steroidhormone (10, 18, 19, 21), zugewandt. Wenn diese Substanzen dauernd in großen Dosen einwirken, weisen sie angeblich eine gewisse endogene Toxizität auf, zum Unterschied von der physiologisch alterierenden Wirkung in angemessenen Dosen. Diese Art der langsamen Intoxikation kann angeblich stufenweise maligne Umwandlung des entsprechenden Gewebes hervorrufen (23). Es wurde die Ansicht vertreten, daß Tumoren endokriner Drüsen bei Versuchstieren auf Gleichgewichtsstörung zurückgehen (41). Gonadotrope Hormone können ein Faktor bei der Entstehung maligner Geschwülste der Testes und Ovarien sein, ein chromophobes Hypophysenadenom wurde bei Mäusen nach großen Östrogenmengen beobachtet; ähnliche Zusammenhänge wurden beim Auftreten von Geschwülsten der Nebennierenrinde und Hyperplasie dieses Organs gesucht. Gesteigerte östrogene Stimulierung ist von besonderer Bedeutung bei der Entstehung und Entwicklung präkarzinomatöser Prozesse und der Krebserkrankung der Milchdrüse. Man verfolgte den Östrogengehalt im Blute bei verschiedenen Zuständen sowohl bei normalen Frauen als auch bei Patientinnen mit Mastopathie und Fibroadenom, ferner mit Karzinom der Milchdrüse. Ein erhöhter Gehalt ist jedoch offenbar nicht die grundlegende Bedingung für die Entstehung und Entwicklung der Krebserkrankung (36).

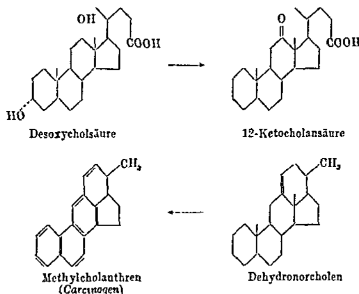
Beim Studium des Einflusses verschiedener Steroide auf die Zellteilung in Gewebskulturen (2) wurde festgestellt, daß lediglich ungesättigte Steroide Mitosestörungen hervorrufen (33). Dirscherl und Breuer (35) untersuchten im Sinne der Warburgschen Theorie von der geschädigten Atmung den Einfluß der Steroidhormone auf die Atmung und Glykolyse des Gewebes der menschlichen Tumoren und stellten fest, daß der Einfluß auf das Krebsgewebe der Brustdrüse nicht einheitlich ist. Dagegen beeinflußten die Androgene in ungünstiger Weise den Metabolismus des Gewebes von Prostatakarzinom in Richtung abnormalen malignen Wachstums, und Östrogene wirkten im entgegengesetzten Sinne. Aus einem benignen Hodentumor, der bei dem Patienten Gynäkomastie auslöste, wurde Östradiol isoliert und die Konzentration dieses Östrogens im Gewebe zu 130000mal höher als normal befunden (72).

30. Hormone und bösartige Geschwülste

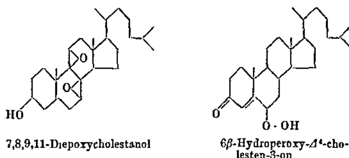
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bei 6β-Hydroperoxy-Δ^4-cholesten-3-on festgestellt, das aus Cholestenon durch Einwirkung von Sauerstoff in Hexanlosung gebildet wird. Es wurde dann untersucht, ob Antioxydantia die Resistenz gegenuber spontaner Karcinogenese erhöhen konnen.



7,8,9,11-Diepoxycholestanol

6β-Hydroperoxy-Δ^4-cholesten-3-on

Ferner wurden die karzinogenen Eigenschaften der Produkte nach der Pyrolyse von Cholesterin bei 360° C untersucht (37), Methylcholanthren konnte jedoch nach der Reaktion nicht festgestellt werden. Auch wurden Versuche zur Überfuhrung der den Nebennierenrindenhormonen ahnlichen 17α-Oxy-20-ketosteroide in D-Homosteroide und 3-Methylchrysenderivate unternommen (45). D-Homoandrostan wurde als nicht karzinogen, das dargestellte 3-Methylchrysen als schwach aktiv befunden.

In der Beziehung der Hypophyse und ihrer Hormone zu den bosartigen Geschwulsten wurden einige Zusammenhange aufgefunden, die vor allem die Sekretion des Vorderlappens betreffen. Bei hypophysektomierten Ratten war Verlangsamung des Wachstums experimenteller Geschwulste zu beobachten (25, 69, 79). Ein gewisser

Nach Kastration bestimmter Stämme von Mäusen, bei denen spontan Milchdrüsenkarzinom auftrat, beobachtete man Abnahme der Zahl der betroffenen Tiere, wenn die Kastration zu einer bestimmten Zeit erfolgte. Später war die Wirkung der Operation auf das Auftreten des Karzinoms statistisch geringer, im Alter von 8 bis 10 Monaten völlig ohne Einfluß (67). Durch wiederholte Östrogendosen ist es gelungen, Mammakarzinom bei männlichen Mäusen hervorzurufen, bei denen es normalerweise nicht spontan auftrat (57). Im Vergleich zu Mäusen sind *Ratten und besonders* Kaninchen, Hunde und Affen gegenüber dieser Wirkung der Östrogene viel widerstandsfähiger. Östrogenzufuhr bewirkte ferner häufigeres Auftreten von Uteruskarzinom und Lymphosarkom bei Mäusen.

Lipschutz (64) beobachtete nach längerer Östrogenzufuhr bei Meerschweinchen benignes fibroides Wachstum des Uterusgewebes und bei kastrierten Männchen Prostatatumoren. Östrogene sind der provozierende Faktor bei zystischen von Uterus*karzinom begleiteten Hyperplasien des Endometriums* (68, 87, 97). *Die heutigen* Anschauungen stimmen im großen und ganzen darin überein, daß Östrogene nicht als ausgesprochene cancerogene Substanzen zu betrachten sind (46, 96). *Nicht einmal* große chronisch verabreichte Hormondosen riefen nach verschiedener Applikation *regelmäßig bösartige Geschwulste hervor*, wie dies bei wirklichen Karzinogenen der Fall ist. Dennoch ist bei der Anwendung von Östrogenen Vorsicht geboten; sie können nämlich gemeinsam mit anderen Faktoren durch Reizwirkung auf verschiedene Gewebe an der malignen Entartung mitbeteiligt sein.

Man suchte nach einem allgemeineren Zusammenhang zwischen den Steroiden und verschiedenen polyzyklischen karzinogenen Kohlenwasserstoffen. Inhoffen (55) behandelte die Aromatisierungsreaktion in der Reihe der Gallensäuren, die in vivo zur Entstehung von Kohlenwasserstoffen des Methylcholanthrentyps führen könnte, bei welchen die karzinogene Wirkung bekannt ist. Methylcholanthren wurde synthetisch aus Desoxycholsäure bereits im Jahre 1933 hergestellt (34, 95) — (s. S. 611).

Bisher bleibt die Frage ungeklärt, ob Karzinogene vom Methylcholynthrentyp im Organismus tatsächlich aus Steroiden und unter welchen Umständen sie gebildet werden, gegebenenfalls ob sie unter Mitwirkung der Darmmikroflora entstehen.

Butenandt und Dannenberg (31) untersuchten verschiedene methylierte Cyclopentanphenanthrenderivate perkutan und als Injektionen auf die kanzerogene Aktivität bei Mäusen; nur vier Substanzen waren nach Auftragen auf die Haut aktiv. Die wirksamen Substanzen tragen die Methylgruppe im Molekül in den Stellungen, die dem angularen Methyl der Steroide unmittelbar benachbart sind.

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Ein Oxydationsprodukt konnte 7,8,9,11-Diepoxy-cholestanol von analog… mit kanzerogenem *Vinylcyclohexen-diepoxyd sein. Carcinogene* Aktivität wurde auch

an Phospholipiden und Phosphor der Fraktionen RNA und DNA in den Kulturen maligner menschlicher Zellen (61). Die Verwertung der Glucose des Mediums war nur wenig erhöht, der Ketosaurenspiegel war wahrend des Wachstums erniedrigt. Mausen des Stammes CFW wurden Zellsuspensionen von Sarkom 180 und 37 inokuliert und 3 Tage hindurch Globin-Zink-Insulin in der Menge von 0,5—2 IE verabfolgt. In den Proben des peritonealen Exsudates beobachtete man nach 4—5 Tagen verminderte Zahl der Geschwulstzellen und einen niedrigeren Prozentsatz der Mitosen als bei den Kontrolltieren. Die Wirkung des Insulins wurde durch wiederholte Injektionen von Glucose aufgehoben (43). Bei Versuchen mit Melanom S91 konnte festgestellt werden, daß die Zellmitochondrien starke glykolytische Aktivität besitzen; Insulin beeinflußt die Regulation der Glucosephosphorylierung direkt in den Mitochondrien (50).

Mit *Adrenalin* prufte man die Hemmung spontaner und implantierter Sarkome bei Mausen (63). Wenn Adrenalin gleichzeitig bei der Implantation verabreicht wurde, trat bei 60 % der Ratten kein Tumor auf, bei spaterer Zufuhr ist jedoch das Verhaltnis bereits bedeutend schlechter.

Steroidhormone wirken in einigen Fällen gunstig lokalisierter Tumoren hemmend auf die maligne Wucherung und schränken das Wachstum dieser Neubildungen ein (3, 5, 27, 29, 54, 84, 92). Diese Wirkung erzielt man durch spezifisches Antagonisieren der im Organismus gebildeten Hormone, die normalerweise vermehrtes Wachstum des entsprechenden Gewebes bewirken. Um den Einfluß der eigenen Hormone im Organismus auszuschalten, wird auch Kastration, eventuell auch Adrenalektomie angewandt, da auch die Nebennieren eine bestimmte Menge von Steroiden mit der Wirkung von Sexogenen produzieren. Es wurde auch der Einfluß von Hypophysektomie oder Hemmung der Abscheidung von Gonadotropinen durch Einwirkung radioaktiver Substanzen oder Hypophyseninhibitoren gepruft. Alle diese Eingriffe fuhren jedoch nur langsameres Fortschreiten bis zur volligen Autonomisierung der Geschwulst herbei, bei der bereits jedwede Reaktivitat auf Hormone verlorengeht.

Sehr eingehend beschäftigte man sich mit der Hormontherapie von Brustkrebs (1, 11, 39, 42, 58, 59, 74) Die Hormontherapie fuhrt hier meist zu vorubergehenden, dennoch therapeutisch wertvollen Ergebnissen. Androgene kommen am besten in Form von Praparaten mit prograhierter Wirkung in der Dosis von 50—100 mg 3mal wochentlich bei Patientinnen vor der Menopause zur Anwendung, nach der Menopause ist angeblich die Verwendung von Östrogenen vorteilhafter, z. B. von Diathylstilbostrol in Dosen von 10—15 mg taglich. Mit Androgenen wurde subjektive Besserung bei 40—76 % der Falle, mit Östrogenen in 33—38 % der Falle festgestellt. Bei Knochenmetastasen bewährte sich Testosteron (71). Nach den Veränderungen der Ca¨-Ausscheidung durch den Harn kann bereits innerhalb weniger Tage geschlossen werden, ob die angewandte Therapie geeignet ist (88).

Neuerdings wird besonders die Hemmwirkung der Sexogene auf die Hypophyse bei der Beeinflussung von Brustdrusengeschwulsten hervorgehoben (58, 81). Man

Zusammenhang wurde namentlich mit der Sekretion des somatotropen (Wachstums-) Hormons gesucht (14). Diese Beziehungen sind jedoch vorläufig nicht ganz klar. Moon (73) beobachtete nach wiederholten Injektionen von Wachstumshormon bei weiblichen Ratten die Entstehung neoplastischer Zellen in verschiedenen Geweben, keineswegs jedoch regelmäßig. Bei Mäusen des Stammes C3H mit transplantiertem Tumor war nach Somatotropinzufuhr Gewichtszunahme und nicht Kachexie wie bei den Kontrolltieren zu beobachten, auch *wenn das Tumorwachstum beschleunigt wurde* (86). Eine Übersicht des Einflusses von Somatotropin und ACTH auf das Wachstum exp. Tumoren gab Reid im Jahre 1954 (14).

Die Menge der sezernierten Hormone oder ihrer Stoffwechselprodukte kann auch als diagnostischer Behelf bei der Unterscheidung verschiedener Tumoren dienen. Steroidhormone im Zusammenhang mit den Geschwülsten der Nebennierenrinde werden noch später erwähnt werden; hier seien die malignen Geschwülste des Plazentagewebes — *Chorionepitheliome* — angeführt, bei denen die innere Sekretion erhalten bleibt. Bei Fällen von Chorionepitheliom ist die Menge Choriongonadotropin im Harn auch über das während der Gravidität übliche Maß stark erhöht. Die Bestimmung dieses Hormons dient zur Diagnose dieser Geschwulst.

Bei der Untersuchung des Einflusses der *Schilddrüse* und der Thyroninhormone auf das Tumorwachstum wurden oft völlig widersprechende Ergebnisse erzielt. Bereits im Jahre 1930 wies Zakrzewski nach, daß ein transplantierter Tumor in Gegenwart von Schilddrüsenextrakten rascher wuchs. Mit Thyroxin wurden später eher umgekehrte Ergebnisse erhalten. Man verfolgte den Einfluß von Thyroxin auf Wachstum und Entwicklung induzierter und transplantierter Geschwülste bei Mäusen und Ratten (44), ferner auf maligne Zellen in Kulturen in vitro, soweit es sich um Stoffwechselveränderungen unter dem Einfluß von Thyroxin und Trijodthyronin handelt (62). Es wurde Verlangsamung des Wachstums der Zellen festgestellt, und ohne daß ihre Zusammensetzung beeinflußt wurde, war die Verwertung der Glucose gesteigert. Auch Analoge von Thyroxin und Trijodthyronin mit Essigsäureseitenkette, die den Stoffwechsel bei thyreoidektomierten Ratten anregen, wurden zur Beeinflussung der Glykolyse bei Zellen von ascitischem Tumor in vitro untersucht (48).

Beim eigentlichen Schilddrüsenkarzinom sind in der Regel noch einige funktionelle Eigenschaften des normalen Drüsengewebes erhalten, insbesondere die Fähigkeit der Jodspeicherung; es kann daher durch radioaktives in großen Dosen verabreichtes Jod beeinflußt werden. Es tritt gewissermaßen eine „physikalische Thyreoidektomie" ein. Die Metastasen des endokrin aktiven Schilddrüsenkarzinoms sind jedoch zweierlei Art, bei manchen ist die Fähigkeit der Jodspeicherung erhalten geblieben, bei andern wiederum nicht. Die letztgenannten werden daher durch Therapie mit radioaktivem Jod J^{131} nicht beeinflußt.

Ähnlich wie bei Thyroxin wurde auch der Einfluß von *Insulin* auf den Stoffwechsel maligner Zellen untersucht. 0,1 IE Insulin in 1 ml bewirkte Steigerung des Gehaltes

Walker-Karzinoms auch Stilböstrol angewandt und hierbei zytotoxische Wirkung festgestellt, was auf den direkten Einfluß des Östrogens auf die Zellteilung deuten wurde (80).

Gewisse antitumorigene Eigenschaften wurden bei Progesteron und einigen *Corticoiden* (65, 70) beobachtet. Die antifibromatogene Aktivität wurde bei einer ganzen Reihe dieser naturlichen sowie synthetischen Steroide verglichen. Cortexon ubte in fast allen Fällen hemmende Wirkung auf die Entstehung von Nebennierentumoren bei kastrierten Mausen aus (52). Bei Ratten mit transplantierten Geschwülsten und auch bei Menschen mit malignen Tumoren war oft gleichzeitige Nebennierenrindeninsuffizienz zu beobachten; daher untersuchte man auch die Beziehung des Tumorwachstums zur Tatigkeit dieser Drüse (53), ferner zum Stoffwechsel der Rindenhormone (85).

Hemmwirkung gegenuber implantierten Tumoren wurde auch bei Cortison sichergestellt (13, 49), insbesondere wenn das Hormon in einem kurzen Intervall nach Implantation der malignen Zellen zugefuhrt wurde Diese Ergebnisse wurden an Mausen, aber auch am Walker-Sarkom der Ratten erzielt. Besserung des Gesamtbefindens beobachtete man nach Zufuhr von Cortison bei Patientinnen nach Operation von Brustkarzinom, wo ausgedehnte Metastasen in den Knochen aufgefunden wurden (47). Die Wirkung war jedoch nur von vorubergehender Dauer. Die Corticoide wurden auch bei Patientinnen mit Karzinom des Cervix uteri angewandt, und zwar jeden zweiten Tag 10 mg Cortiron (DOCA) bei gleichzeitiger Bestrahlungstherapie mehr als 3 Wochen hindurch (82). Hemmaktivitat des Cortisons gegenuber dem Karzinom 755 wurde bei Versuchsmausen beobachtet (24), hingegen traten beim Tumor T 150 bei Mäusen nach Cortison ofter Metastasen auf (26), ähnlich auch bei experimentellen Rattentumoren (60) Kombinierte Darreichung von Cortison und Stickstofflost bewährte sich nicht. Cortison und besonders Prednison wurden auch bei Leukamien in der Tagesdosis von 30—50 mg angewandt (76). Diese bewirkten Temperaturabnahme und Besserung des Gesamtbefindens bei einer Reihe von Patienten mit verschiedenen Arten von Leukamie.

Ähnlich wie Cortison wurde in Fallen vorgeschrittenen Karzinoms auch *ACTH* (16) angewandt, und zwar in Tagesdosen von 100—200 mg 2—6 Wochen hindurch Bei einem Teil der Patienten war vorubergehende Regression, bei den ubrigen nur vorubergehende Besserung des Gesamtbefindens zu verzeichnen (89)

Eingehend suchte man auch nach den *Beziehungen* zwischen dem Wachstum bösartiger Geschwulste und den Spiegeln der Hormone und ihrer Stoffwechselprodukte in den Korperflussigkeiten (8, 15). Hinsichtlich der Ausscheidung von 17-Ketosteroiden im Harn von Patienten, die an Krebserkrankung verschiedener Organe litten, wurde keine systematische Modifizierung der Ausscheidung festgestellt, mit Ausnahme der Falle von Nebennierenrindenkarzinom (75) Die Exkretionswerte werden von andern Faktoren, wie z. B Alter, Geschlecht, Ausmaß der Diurese u ä.m.,

empfiehlt, die Operationstherapie mit der Röntgenkastration zu kombinieren (28, 77, 93); die Hormontherapie kommt sonst bei inoperablen Fällen zur Anwendung und ist als palliative Therapie zu betrachten. Ähnlich wie Testosteron wurde neuerdings Androstan-17β-ol-3-on (*Stanolon, Neodrol*) in der Tagesdosis von 100 mg i m. verwendet. Bei Mammakarzinom wird die Hormontherapie zumeist erst dann eingeschaltet, wenn die übrigen Behandlungsweisen versagen (90).

Östrogene bei der Behandlung von Prostatakarzinom wurden von Huggins (9) bereits im Jahre 1941 angewandt. Die Tumoren des Hinterlappens dieser Drüse pflegen bösartig zu sein, während die des Vorderlappens zumeist gutartig sind. Bei längerer Verabfolgung des Östrogens atrophiert das Vordersegment, das Hintersegment behält jedoch lange seine Funktion bei. Ein charakteristisches Kennzeichen bei Prostatakarzinom ist die Erhöhung des Spiegels der sauren Phosphatase im Blutserum. Androgene bewirken Steigerung des Enzymgehaltes im Serum, nach Östrogenen tritt Abnahme ein. Das Versagen der Östrogentherapie ist nach Huggins zweifacher Art. Einmal tritt es bei Patienten nach der Kastration ein, bei denen Androgene in größerer Menge aus den Depots außerhalb der Gonaden, d. h. den Nebennieren, produziert werden, zum andern in Fällen, bei denen die Wirkungen verwickelter und bisher noch nicht hinreichend geklärt sind. Manche Karzinome sind so dedifferenziert, daß sie auf hormonale Behandlung überhaupt nicht reagieren. Es hat also den Anschein, daß Neubildungen in manchen Fällen „autonom" sind, in andern häufigeren Fällen wird jedoch ihr Gewebe einseitig durch Hormone gesteuert.

Neuerdings verwendet man bei Prostatakarzinomen Diäthylstilböstroldiphosphat, das unter der Bezeichnung *Honvan* (30, 40) in den Handel kommt, ferner Chlortrianisen (TACE), das durch Speicherung im Fett des Organismus verlängerte Wirkungsdauer gewährleistet (78). Die Tagesdosen betragen bei diesem Präparat ungefähr 24 mg. Bei der Therapie mit Diäthylstilböstrol wurden bereits mehrfach Metastasen in den Brustdrüsen beobachtet (56, 83). Anscheinend hängt die Verwendbarkeit des Östrogens bei der Applikation in der Behandlung des Prostatakarzinoms nicht von seiner Aktivität im klassischen Test nach Allen und Doisy ab, denn schwache Östrogene erwiesen sich oft als geeigneter, so war z. B. α-Bromtriphenyläthylen besser als Diäthylstilböstrol (32).

Erfahrungen mit der Verwendung von Äthinylöstradiol bei Prostatakarzinom veröffentlichte Weber (94); bei neun Fällen wurde subjektive und auch objektive Besserung festgestellt, zwei Fälle sprachen nicht an. Nach Anwendung von Progesteron (91) beobachtete man klinische Zustandsbesserung, die durch den Phosphatasengehalt bei 70 % der Kranken kontrolliert wurde. Ferner wurden vorläufig sehr gute Erfahrungen mit dem Methyläther des 16-Ketoöstradiols festgestellt, das nur $^1/_{400}$ der östrogenen Aktivität des Diäthylstilböstrols besitzt. Huffman und Katzberg stellten fest, daß das erwähnte Steroid auch in äußerst starker Verdünnung die Metaphase der Zellteilung stark beeinflußt (22). Experimentell wurde bei Zellen des

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stark beeinflußt. Nur bei den Geschwülsten der Nebennierenrinde können aus den Befunden gewisse Regelmäßigkeiten abgelesen werden. Mittels Papierchromatographie wurden die Steroide im Harn einer Patientin mit virilisierender Nebennierenrindengeschwulst festgestellt, auch wurden die Steroide im Gewebe des Tumors nach seiner Entfernung ermittelt und vor allem eine größere Menge Cortisol und Cortison festgestellt (66).

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HANDBUCH DER PAPIERCHROMATOGRAPHIE

2 Bände

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